

Antigen Presentation in Autoimmune Disease

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Dedication

I would like to dedicate this work to my family – they may have thought me mad, stressed or in need of a proper job, but they never stopped being proud of my achievements and they never stopped supporting my choice of what I wanted to do. I could not have done this without them.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

Naomi Jane Marshall

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The aim of my project was to examine the extent to which endogenous expression of a largely renal-specific antigen influences the repertoire in adulthood of autoreactive T cells specific to that antigen.

The renal-specific antigen, human $\alpha 3(\text{IV})\text{NC1}$, is the target of autoimmune attack in Goodpasture's disease. This protein was expressed and purified in recombinant (using bacterial and mammalian cell expression systems) and purified in native (extracted from human tissue) forms. Transgenic mice were generated that express HLA-DR15 (associated with Goodpasture's disease) as their sole MHC class II molecule, and for which $\alpha 3(\text{IV})\text{NC1}$ can be endogenous or exogenous. The CD4 T cell responses of these mice were then tested following immunisation with $\alpha 3(\text{IV})\text{NC1}$.

In mice with endogenous expression of $\alpha 3(\text{IV})\text{NC1}$ there were no consistent detectable proliferative T cell responses to any $\alpha 3(\text{IV})\text{NC1}$ peptides in a set of overlapping peptides representative of the entire sequence. In the mice lacking endogenous $\alpha 3(\text{IV})\text{NC1}$ there were consistent responses to the peptide $\alpha 3(\text{IV})\text{NC1}$ 136-150. This contains part of the peptide recognised by the most abundant autoreactive T cells in patients with acute Goodpasture's disease. Therefore, the T cell responses seen in man to an endogenous (auto)antigen have similar fine specificity to those seen in mice responding to the same protein as a foreign antigen. This is surprising as one might expect self-tolerance in man to be most secure to such

dominantly presented and immunogenic (in HLA DR15 mice) self peptides. However, recent work suggests that the peptide most commonly presented in humans is normally destroyed during antigen processing, giving a possible explanation for the lack of tolerance.

Future work should study why tolerance is ineffective to this particular peptide, whether tolerance can be reinforced, these questions could be addressed using a transgenic mouse model that develops Goodpasture-like pathology. In addition, how processing is defective in Goodpasture's disease could be explored by making antigen presenting hybridomas from patient samples or from the transgenic mouse line described within this thesis.

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Abbreviations

α - Alpha

$\alpha 3(IV)NC1$ - $\alpha 3$ chain of type IV collagen, non-collagenous domain

ADP – adenosine diphosphate

AEP – asparaginyl endopeptidase

Ag - antigen

AIRE – autoimmune regulator

Alk phos - alkaline phosphatase

APC – antigen presenting cell

β - Beta

β Me - β -mercaptoethanol

B cells – B lymphocytes

BCIP – 5-bromo-4-chloro-3-indolyl phosphate

BCR – B cell receptor

bp – base pairs

BrdU – bromodeoxyuridine

BSA – bovine serum albumin

ccpm – calculated counts per minute

CD – cluster of differentiation

CDR – complementarity determining region

CFA – complete Freund’s adjuvant

CLIP – class II-associated invariant-chain peptide

CNBr – cyanogen bromide

CO₂ – carbon dioxide

COLIVA3 – gene encoding α 3 chain of type IV collagen

con A – concalavalin A

CTL – cytotoxic T lymphocytes

CTLA-4 – T cell transmembrane signalling molecule

DAPI – 4,6-diamidino-2-phenylindolecarbamide

DMEM – Dulbecco’s modified Eagle’s media

DMSO – dimethyl sulphoxide

dNTPs - deoxyribonucleotides

DTD – diphtheria toxoid

DTT - Dithiothreitol

E. coli – Escherichia coli

EAE – experimental allergic encephalomyelitis

EDTA – ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbant assay

ER – endoplasmic reticulum

Fab – Fragment, antigen binding, of immunoglobulin

FISH – Fluorescent *in-situ* hybridisation

FITC – fluorescein isothiocyanate

g – relative centrifugal force

GFP – green fluorescent protein

h – hour(s)

H – heavy chain (of T cell receptor)

^3H - tritium

HAT - Hypoxanthine- Aminopterin- Thymidine

HBSS – Hank’s buffered saline solution

hCG – human chorionic gonadotropin

HEL – hen egg lysozyme

Het – heterozygous/ heterozygote

HI-FBS – heat-inactivated foetal bovine serum

HLA – human leukocyte antigen

homo – Homo sapiens (human)

HPLC – high performance liquid chromatography

HT - Hypoxanthine- Thymidine

hu α 3 – purified human α 3(IV)NC1

IAA – iodoacetic acid

IC₅₀ – concentration at which 50% target molecules are inhibited

ICAM – intercellular adhesion molecule

IFA – incomplete Freund’s adjuvant

IFN- γ - Interferon- γ

IgG – Immunoglobulin G

Ig κ - Immunoglobulin kappa light chain

Ig λ - Immunoglobulin lambda light chain

Ii – invariant chain

IL – Interleukin

IPTG - isopropyl β -D-1-thiogalactopyranoside

kDa - kilodalton

KLH – keyhole limpet haemagglutinin

KO – knockout

L – litre

LacY – lac permease gene

LB – Luria-Bertani

LFA – leukocyte functional antigen

LPS - lipopolysaccharide

μ - micro

m - milli

M - molar

MBP – myelin basic protein

MgCl₂ – magnesium chloride

MHC class I – major histocompatibility complex class I

MHC class II – major histocompatibility complex class II

MIIC – MHC class II compartment

min – minute(s)

mRNA – messenger RNA

MS – mass spectrometry

mus – *Mus musculus* (mouse)

n - nano

NaCl – sodium chloride

NBT – nitro blue tetrazolium

NOD – non-obese diabetic

PAA – prostate-associated antigens

PBMCs – peripheral blood mononuclear cells

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PE – phycoerythrin

PEG – polyethylene glycol

rh α 3 – bacterial recombinant human α 3(IV)NC1

RhD – Rhesus D antigen

s – second(s)

SDS – sodium dodecyl sulphate

SDS PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis

SI – stimulation index

sIgG – surface immunoglobulin G

SNP – single nucleotide polymorphism

T cells – T Lymphocytes

TBE – Tris Borate EDTA

TCR – T cell receptor

TeNT – Tetanus neurotoxin

Tg - transgene

TGF- β - Transforming Growth Factor- β

Treg – regulatory T cell

TTCF – Tetanus toxoid C fragment

TTD – Tetanus Toxoid

V – Variable chain (of T cell receptor)

WT – Wild Type

YFP – Yellow Fluorescent Protein

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Chapter 1 – General Introduction

The main aim of this project was to determine CD4 T cell immune responses to a known and well-characterised kidney autoantigen, the non-collagenous domain of the $\alpha 3$ chain of type IV collagen ($\alpha 3(\text{IV})\text{NC1}$), in the presence and absence of tolerance to that antigen. It was clear from previous work that T cells specific for this autoantigen occurred in healthy individuals as well as those with acute autoimmune disease (1-3), raising questions as to the nature of T cell tolerance to this tissue-restricted basement membrane protein. $\alpha 3(\text{IV})\text{NC1}$ is expressed in the human thymus (4), so some element of central tolerance would be expected, but clearly that tolerance was far from complete. Other data indicating interleukin (IL)-10 production from $\alpha 3$ -specific T cells pointed to a role for peripheral tolerance and T cell regulation. This project was conceived to examine the completeness of tolerance to $\alpha 3(\text{IV})\text{NC1}$ and its robustness in the face of immunisation with $\alpha 3$.

1.1. – CD4 T cell responses and epitope specificity

When a CD4 positive T cell is stimulated, it proliferates and releases IL-2 in order to both propagate clonal expansion and to attract other white blood cells from the lymphatic system to the site of T cell activation. This is a part of the cellular immune response and plays a role in eliminating extracellular threats (5).

However, antigen alone does not stimulate CD4 T cells. These cells will only respond to processed antigen presented on the surface of antigen presenting cells (APCs). APCs can take up antigen by phagocytosis (macrophages and dendritic cells) or through receptor binding interactions (B cells). This antigen can be then processed into fragments, bound to major histocompatibility complexes (MHC) class II binding grooves, and presented on the APC surface (6). CD4 T cells respond to a specific combination of the MHC class II molecule and the peptide within the binding groove. The CD4 T cell response is therefore focused on fragments of antigen known as epitopes (5).

This is in marked contrast to the immune responses of B cells, another crucial element of the cellular immune response. The B cell receptor (BCR) recognises specific conformations on proteins. The receptor binds the whole antigen, so tertiary and quaternary structural features of the protein are very important for enabling B cell recognition (7, 8). Once the antigen has been bound to the receptor, the receptor-antigen complex is endocytosed and enzymatically processed in lysosomes. The peptide fragments are mixed with MHC class II molecules and those that bind to a peptide-binding groove on the MHC class II are presented on the cell membrane (6).

The antigen receptors on B cells are multiprotein complexes made up of variable antigen-binding chains – the heavy and light immunoglobulin chains. The invariant part of each chain is associated with specific accessory proteins. These have two major functions: to transport the receptor to the cell surface; and to initiate signalling when the receptors bind an extracellular antigen (9). Each B cell only produces one binding domain. When antigen binds to surface immunoglobulin, the

signalling pathways activate and turn on the genes for protein production with the result that the cell is activated. The surface immunoglobulins have an identical antigen binding region to the secreted immunoglobulins produced by the activated cell, but are anchored to the cell membrane through the carboxy termini of the paired heavy chains (6).

Studies in man using peripheral blood mononuclear cells (PBMCs) and studying T cell responses to tetanus toxoid (TTD) and diphtheria toxoid (DTD) have found that T cell responses in most individuals include certain common, so-called “universal epitopes”. These epitopes are thought to be able to cause an immune response in cells with major responses to a different antigen, so contain features similar to multiple pathogens. Work studying TTD and DTD as a whole antigen versus peptide fragments showed similar orders of magnitude in immune response (10), but also that 8-29% of the toxoid specific CD4 T cells responded to the TTD, while 28-51% responded to the DTD. These percentages also support the concept of universal antigens as all but three of the candidates taking part had had booster injections more than four years before the study. Therefore, they were unlikely to be generating an immune response to either TTD or DTD prior to extraction of the PBMCs. It is also unlikely that all the responsive cells were primed to the same antigen, suggesting that the responses were to antigenic peptides that were somehow comparable to the original priming antigenic fragment (10).

1.1.1. – Subsets of CD4 positive T cell responses

CD4 positive T cells have a number of different responses to antigen and these can be distinguished by the cytokines released by the activated T cell. The categorisation of these responses increases in complexity as more is discovered about

the range of responses exhibited by Th cells. While acknowledging this complexity, there are two broad classes of Th cell response (Th1 and Th2) that remain useful in distinguishing between different outcomes for T cell activation. Th1 responses are characterised by interferon- γ (IFN γ) and generally associated with destructive responses such as viral infections. In contrast, Th2 responses are characterised by interleukin (IL)-4, IL-5, IL-6 and IL-10 and generally associated with humoral immune responses, although asthma is also characterised by the production of Th2 cytokines. An additional major class of Th cell is regulatory T cells (Tregs) (11) which aid peripheral tolerance and maintain immunological homeostasis. Additional, more recently described, subsets are Th9 and Th17 cells (12). The nature of immune responses depends on the balance of the different Th subsets involved, which in turn depends on probably numerous factors. These may include co-stimulatory molecules, the site of antigen-presentation, peptide density and binding affinity (high density favours Th1, low density favours Th2), antigen dose and the host genetic background (5). It is now known that the differentiation decision is mainly governed by cytokines in the surrounding microenvironment and the strength of the interaction between the T cell antigen receptor with antigen (13).

Th1 responses promote cellular immunity against intracellular organisms (14) and cell-mediated inflammatory reactions, they are also predominant organ-specific immune disorders, acute allograft rejection, and multiple sclerosis, amongst other things. The Th1 response is characterised by release of IFN γ . These promote the production of IgG2a opsonising and complement-fixing antibodies, macrophage activation, antibody-dependent cell-mediated cytotoxicity and delayed-type

hypersensitivity. The major cytokine produced by Th1 cells is IFN γ which causes macrophage activation, but also actively inhibits proliferation of Th2 cells (5).

Th2 T cells produce different cytokines and initiate a different type of T cell response. Th2 responses are characterised by secretion of IL-4, IL-5, IL-6 and IL-10. These cells provide optimal help for humoral immune responses, including IgG1 and IgE isotype switching and mucosal immunity, stimulation of mast cell and eosinophil growth and synthesis, and IgA synthesis. They are typified by the production of IL-4 and IL-5 with IL-6, IL-9, IL-10 and IL-13 also being produced. In comparison with Th1 responses, Th2 cells are associated with strong antibody and allergic responses. Overall, Th2 responses are classified as protective as they negate Th1 responses but do result in immune responses. In particular, asthma has been linked to Th2 T cell responses (15). However, Th2 responses also promote long-term recognition of antigen by initiating antibody responses and therefore the existence of memory B cells, increasing responses to a future threat. The cytokines produced by Th2 cells (IL-4 and IL-5) activate mast cells, B cells and eosinophils, resulting in the production of antibodies including IgE. In addition, Th2 cells produce IL-4 which actively inhibits the production of IFN γ by Th1 cells, limiting the Th1 response – a reciprocal relationship as Th1 negate Th2 responses, while Th2 negate Th1 responses. Some work on Th1 and Th2 cells has shown that once a cell is in either of these lineages, it is terminally differentiated (14, 16). However, it has also been shown that TGF- β in the presence of IL-4 reprograms differentiation of Th2 cells into a population of Th9 cells that secrete IL-9 and some IL-10. IL-4 effectively blocks production of TGF- β -induced Treg cells and results in Th9 cells which have no suppressor function and promote tissue inflammation (17).

Th17 cells produce IL-17A, IL-17F and IL-22 and have been found to have an important role in clearance of extracellular bacteria and fungi, especially at mucosal surfaces. Differentiation into Th17 cells requires retinoid-related orphan receptor γ t, a transcription factor induced by TGF- β in conjunction with the proinflammatory cytokines IL-6, IL-21 and IL-23. These then result in Stat3 phosphorylation (18). As with Th1 cells, Th17 cells play a critical role in organ-specific autoimmunity (19).

Treg cells have an essential role in maintaining immune homeostasis. They regulate the effector T cells (Th1, Th2 and Th17 are all effector T cell responses) and prevent potentially pathogenic effects through a variety of mechanisms (20, 21). Treg cells are characterised by expression of the forkhead transcription factor, FoxP3, which plays a critical role in specifying and maintaining the functional programme of these cells. The Treg cell population is also complex, but has to date been split into two categories: the so-called naturally occurring CD4⁺ CD25⁺ Treg that develop in the thymus (nTreg), and the TGF- β -induced Tregs (iTregs) produced in the periphery. Both of these sets of cells are believed to have a role in maintaining peripheral tolerance and preventing autoimmunity, but distinct roles for each have yet to be fully dissected *in vivo* (22-24). It has been shown that nTregs require T cell receptor ligation and cell-cell contact for effective immunosuppression (25), while iTregs secrete cytokines to achieve a similar result (26). Overall, Tregs secrete IL-10 which results in: the suppression of dendritic cells; suppression of Th1, Th2 and Th17 responses; suppression of allergen specific IgE but induction of IgG4; suppression of mast cells, basophils and eosinophils; and suppression of effector T cell migration to tissues. In addition, Treg cells interact with resident tissue cells and

influence remodelling (17). In contrast with Th1/Th2 differentiation, expression of IL-17A by Th17 cells and of FoxP3 by induced Treg cells have been shown to be less stable, increasing the flexibility of their differentiation options (14).

1.1.2. – T cell receptor plasticity and molecular mimicry

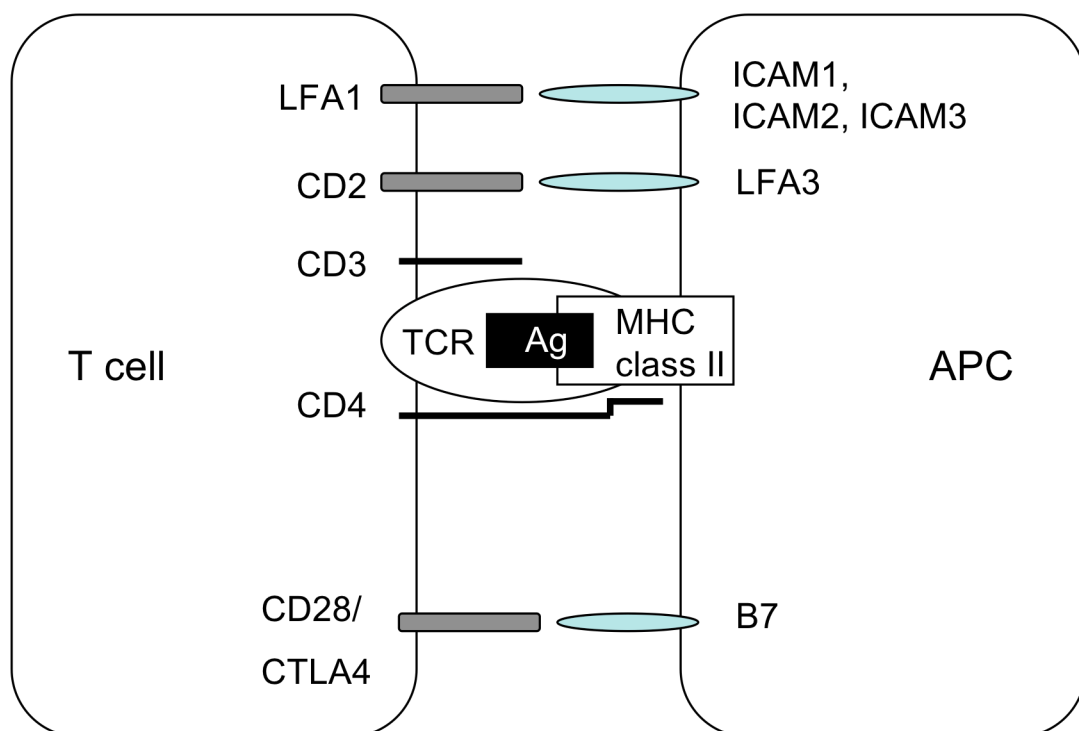
Classical immunology suggests that each T cell has a specific receptor (TCR) that recognises a specific epitope in conjunction with the MHC class II molecule. It has since been found that there is more plasticity in TCR recognition than was originally thought. Experiments using a transgenic mouse line with restricted TCR repertoire revealed that this mouse was able to generate an immune response to all antigenic fragments tested, compared with non-transgenic littermates. This suggests that, despite a more limited TCR, there was no difference in the response specificity profile between transgenic and non-transgenic mice. However, the results from transgenic mice showed a 3-8-fold decrease in the frequency of transgenic T cell responses. These data demonstrate unexpected plasticity in the TCR repertoire (27). Subsequent work has suggested that there are limitations on the degree of plasticity. It has been shown that peptides altered by a single amino acid generated different immune responses compared with unaltered peptide and caused variation in the level of cytokine production (28). In addition, work by Gebe *et al.* has demonstrated that T cells selected on an MHC class II molecule carry the potential for activation on altered self-ligands when encountering antigens presented on a related MHC class II molecule. In individuals heterozygous for the alleles tested (human leukocyte antigen, HLA-DRB1*0401 and DRB1*0404), the possibility of TCR cross-recognition could lead to an aberrant immune response or autoimmunity (29).

The priming of T cells by one antigen, resulting in a sustained response to a different self-antigen is known as molecular mimicry. Wucherpfennig *et al.* have shown that viral peptides can activate T cells specific for myelin basic protein (a candidate autoantigen in multiple sclerosis) (30). Other work by Uemura *et al.* identified candidate epitopes for which mimicry was possible, in the T cell population specific for glutamic acid decarboxylase-65 (implicated in type 1 diabetes) (31). Molecular mimicry is more specific than identification of universal epitopes as specific viral or bacterial antigens have links to a specific autoantigen, whereas universal epitopes, as the name suggests, are more general, bind to the majority of HLA molecules and can be found within multiple antigens (those described above, TTD and DTD).

As are described in more detail below, T cell epitopes are dramatically different from B cell epitopes. T cell epitopes are contained within fragments of an original antigen, usually 12-17 amino acids in length. By comparison, B cell surface receptors recognise whole antigen so recognise conformations, specific structural features, as well as sequence.

Figure 1.1 – Schematic of the trimolecular complex

This schematic illustrates the interaction between CD4 positive T cell, the MHC class II complex and the peptide bound within the binding groove, in conjunction with other cell surface markers required for T cell activation (adapted from Janeway *et al.* (6)). The major interaction illustrated is between the MHC class II molecule, the antigen (Ag) and the TCR. The additional interactions shown are between costimulatory molecules on the APC (right) and T cell (left) required to propagate the stimulatory immune response, resulting in T cell proliferation.



1.2. – Molecular basis of T cell epitope specificity

When a CD4 T cell recognises a peptide in the MHC class II binding site, the binding formed is known as the trimolecular complex and consists of the TCR, the MHC class II molecule (or human leukocyte antigen, HLA) and the peptide fragment (figure 1.1.). The TCR binds in a specific orientation. Each TCR consists of two disulphide-linked polypeptide chains: either α and β ; or γ and δ . The overall features of the structure show that the two chains fold into a structure resembling the Fab region of immunoglobulin (32). Both receptors are associated with a set of five polypeptides, the CD3 complex, and together form the T cell receptor complex. Approximately 90-95% of T cells are $\alpha\beta$ T cells, while the remaining 5-10% are $\gamma\delta$ T cells (thymic T cells and a minor population of peripheral T cells) (5).

1.2.1. – MHC class II binding

Most antigens taken up and processed by antigen presenting cells, including B cells, can be processed into lots of fragments. Not all of these fragments bind the MHC class II binding groove with high enough affinity to remain bound and be transported to the cell surface to be presented to passing T cells. The fragments that do successfully bind and that are presented are potential T cell epitopes. It has been shown that the length of peptide sequence bound by MHC class II molecules is less limited than peptides for MHC class I molecules. Most appear to be around 12 amino acids in length, but they can be longer (MHC class I peptides are 9 amino acids long). These peptides lie within the peptide groove, and are held there by

interactions between the peptide backbone and side chains, and between shallow and deep pockets that lie along the length of the groove (33, 34). The peptides within the binding groove are bound by their backbone and can overhang from either end of the class II binding groove. This means that, theoretically, there is no upper limit for the length of peptide bound by the complex. In practice, most of the longer peptides are trimmed by peptidases to between 12 and 17 amino acids (35, 36).

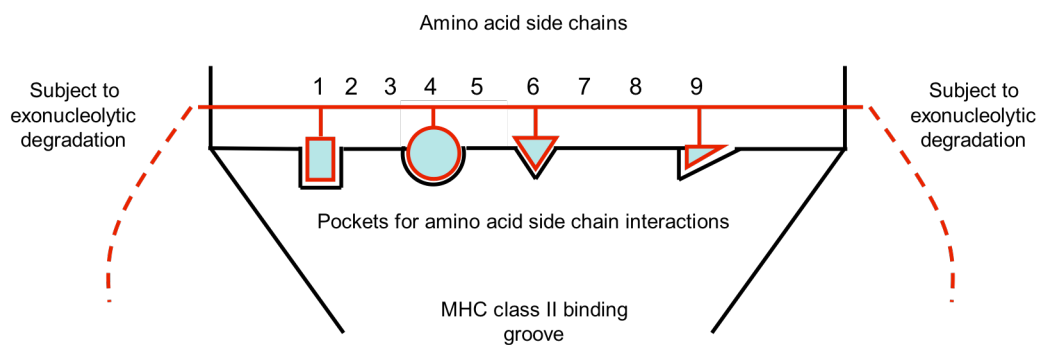
Through X-ray crystallography, it seems that amino acid side chains from residues 1, 4, 6 and 9 of the peptide (corresponding to pockets 1, 4, 6 and 9 of the MHC class II binding groove) are the most important for disrupting the overall binding of the peptide within the groove (37, 38). However, there is a possibility that the MHC class II binding groove may have an overhang at the N-terminal end of the peptide. This was demonstrated by a study in which mice were immunised with either an immunogenic peptide, or a longer peptide containing the immunogenic peptide sequence. In this study, the longer peptide was found to propagate more long-term immune responses and induce immunological memory, while the shorter peptide did not (39). This may be due to the processing required for the peptide binding, or due to the increased flexibility underlying the peptide fragment binding the MHC class II molecule. It could also be a result of the longer peptide containing an overlapping epitope with higher affinity for MHC class II molecules than the short peptide.

1.2.2. – Peptide presentation by MHC class II molecules

The MHC class II molecule is very important in determining which peptides are presented and which remain in the lysosome for further destruction and subsequent recycling of the amino acids. The pockets within the MHC class II

Figure 1.2. – Side chain interactions of the MHC class II binding groove.

This schematic illustrates the binding of a peptide into the MHC class II binding groove and shows pockets with which amino acid side chain interactions reduce affinity for the binding domain (adapted from Milinski *et al.* (40)). As is shown, the pockets are located to accommodate side chains from amino acids 1, 4, 6 and 9 on the peptide chain, while the ends of the binding groove remain open. This potentially enables any length of peptide to bind although these are more usually 12-17 amino acids in length.



binding groove are lined with polymorphic residues that can exert some discrimination and prevent binding of peptides with lower affinity. The arrangement of polymorphic pockets within the binding groove is not as restrictive as the very definite binding groove found in MHC class I molecules, making it difficult to ascertain which specific peptides will bind with the highest affinity to a specific MHC class II molecule. However, by comparing sequences of peptides with known binding ability, certain common features can be determined and permissive amino acids for a specific class II allele can be defined (41-43). This then enables modelling to show how the amino acids of a specific peptide will interact with the MHC class II binding groove. A schematic of the binding groove is illustrated in figure 1.2.

MHC class II molecules without bound peptide are inherently unstable, but the mechanism by which the MHC class II molecule is stabilised by binding peptide is, as yet, unknown. This cannot be limited to specific amino acid residues binding specific regions in the MHC class II binding groove, as this restriction would greatly reduce the variation in peptides that are capable of binding (6).

Various methods for investigating epitope selection by MHC class II molecules have been developed. As has already been described (section 1.1.), whole antigen has to be processed by APCs and presented on their surfaces bound to MHC class II molecules. While an antigen may be processed into dozens of fragments, only a few bind with a high enough affinity to the MHC class II molecules to actually be presented. In particular, work has concentrated on determining the peptide fragments that generate the greatest immune responses when bound within the MHC class II binding groove. These are not necessarily peptides that are presented with

the greatest abundance on the cell surface, but are those that bind with the highest affinity to the MHC class II molecule (44-47). The fragment resulting in the most potent immune responses is classified as the immunodominant peptide as defined by T cell responses (44).

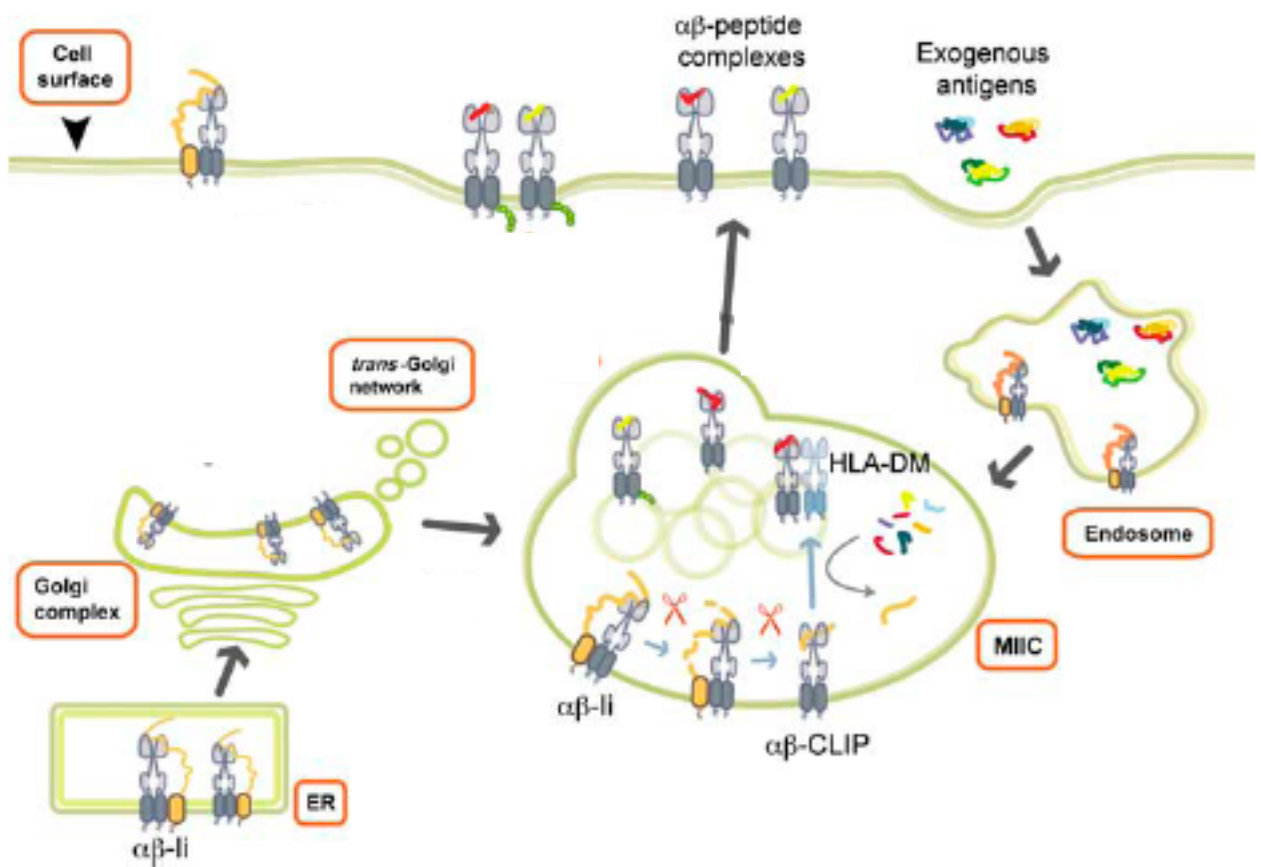
1.3. – Influence of MHC class II on selection of epitopes recognised by T cells

Questions then arise regarding determination of the immunodominant peptide. It is now thought that the process is partly driven by the MHC class II molecule and by the MHC class II “blocking peptide”, class II-associated invariant-chain peptide or CLIP (44, 46). To understand this, more needs to first be understood about the production of the MHC class II molecules. These molecules are polygenic and polymorphic, meaning that there are multiple genes and multiple alleles at these loci. The genes and alleles may not be transcribed equally (for example DRB1 transcription shows a 5-fold excess of mRNA compared to DRB4 (48)). The genetic variation and differing protein expression ensures diversity on an APC surface and is in contrast to both the BCR and the TCR, as BCR and TCR are monotypic molecules. The receptor is unique to the B or T cell, so variation is obtained by cell numbers rather than with variety of surface receptors presented on each cell (49, 50).

When the MHC class II molecule is translated, it could potentially bind any available peptide fragment with sufficient binding affinity within its empty binding groove. This could cause autoimmunity or an aberrant immune response. To

Figure 1.3. – MHC class II production and antigen presentation

MHC class II $\alpha\beta$ heterodimers are assembled in the endoplasmic reticulum, with the Ii fragment in the binding groove. The major route of antigen presentation by MHC class II molecules shows Ii chaperoning the MHC class II molecule directly (black arrow) into the MIIC where Ii is degraded and antigenic fragments bind into the MHC class II binding groove in subdomains of the MIIC prior to transport to the cell surface (blue arrows). Adapted from Rocha *et al.* 2008 (51).



prevent this, the MHC class II molecules are made with the invariant chain (Ii) peptide bound within the binding groove (section 1.3.). This peptide, when the MHC class II molecules coincide with processed antigen within the lysosome, is then cleaved enabling antigen-derived fragments to bind instead (figure 1.3.). Relatively recently, work was published describing how the affinity of a peptide for the MHC class II, when comparable with the affinity of the Ii peptide with the MHC class II, may play a role in determining immunodominance (52, 53). Peptides of high affinity derived from the CLIP and Ii have been shown to compete successfully with those of low affinity but derived from antigen, for binding to newly synthesised MHC class II molecules and thereby favouring their presence in the periphery (37). This supports the notion of dominant capture – a process by which the first, most available and high affinity binding region on the antigen would preferentially bind to the MHC class II molecules. Since the peptide binding groove of MHC class II molecules is open-ended, there is no limit to the length of peptide that can bind. If binding occurs early within the lysosome, the bound peptide would be protected by the groove, while the fragment outside the binding domain is cleaved by external proteases and resulting in a small subset of fragments being presented on the cell surface. This therefore predicts that a single determinant or a small number of determinants would emerge as competitively favoured over other determinants derived from the antigenic fragment.

In summary, peptides that bind MHC class II with some affinity can be presented on the APC surface. A current hypothesis regarding influences affecting peptide presentation is determinant capture. Current evidence supporting determinant capture includes the fact that there is no length restriction on the

peptides binding the MHC class II binding groove; that residues bound within the groove are then protected from further proteolysis; and that the immunogenic peptides are generated and bind the MHC class II molecule within the same compartment (33, 45, 54, 55).

1.4. – Evidence for influences beyond MHC class II on T cell response to exogenous antigen

Affinity for MHC class II molecules is important but affinity alone is not thought to be sufficient to ensure peptide presentation. It has been postulated that influences in addition to peptide sequence may have a role in determining how an antigen is processed and the presentation of processed peptides. These influences have been postulated to include processing differences, MHC structure and competitive binding, availability to bind MHC class II, and quaternary structure and disulphide bonds. All of these are linked to peptide sequence but may add complexity to the processing mechanisms.

1.4.1. – The effect of processing on MHC class II binding by peptides

Work studying immune responses in mouse strains expressing the same class II molecule, but in which there were differential immune responses to immunogenic peptides suggested that the ability to respond to the specific peptide does lie within each strain (56). It has been further hypothesised, using HEL peptide immunisation with subdominant peptides, that low level immune responses can arise by three

mechanisms: a) competition by an immunodominant peptide *in vivo* resulting in the subdominant peptide failing to bind the MHC molecule; b) failure to generate the subdominant peptide during processing; and c) an inherently poor capacity of the T cell repertoire to respond to a particular peptide-MHC complex (57).

1.4.2. – The effect of antigen structure on MHC class II binding by peptides

Early work by Streicher and Berzofsky *et al.* proposed that antigen conformation determines processing requirements by studying four different forms of a specific epitope of myoglobin (amino acids 132-153) (58). These forms included the native antigen, the specific peptide fragment and two conformationally altered forms of the protein. By investigating the T cell recall responses to these proteins, the group found that unfolding the native conformation was a critical parameter determining processing and showed that the epitope within native myoglobin was the least accessible of the four tried. They proposed that processing may be necessary to allow effective presentation of the epitopes. Berzofsky's group subsequently showed that there was evidence of MHC-specific hindering structures on products of processing (59). This was hypothesised as result of examining the recall responses to equine myoglobin and an immunogenic myoglobin peptide in different strains of mice. One strain generated an immune response to both whole antigen and the specific peptide, while the other strain of mouse only generated an immune response to the peptide. Since the mouse strains used express different class II molecules, it was hypothesised that the product of natural processing of the whole myoglobin protein contained sequences flanking the immunogenic peptide, which interfered with peptide binding to the MHC class II molecule or subsequent TCR binding (59).

1.4.3. – The effect of three-dimensional structure and disulphide bonds on MHC class II binding

More recently, it was hypothesised that three-dimensional structure potentially limits the access of endoproteolytic processing enzymes to cleavage sites and of MHC class II molecules to helper T cell epitopes. Data from Dai *et al.* has shown that the immunodominant peptide from bacteriophage T4 Hsp10 is on the N-terminal flank of a mobile loop, which is sensitive to proteolysis. Mutations to stabilise this loop dramatically reduced the immunogenicity of the flanking immunodominant epitope, although the protein retained good overall immunogenicity. They also showed that antisera generated against the stabilised mutant T4 Hsp10 epitopes exhibited increased cross-reactivity. Overall these data support the concept that unstable loops can promote the presentation of flanking epitopes and suggest that deletion of these loops may have implications in improving both the breadth and strength of an immune response (60).

Experiments by Rouas *et al.* using human chorionic gonadotropin (hCG) α - and β -subunits showed that the α - subunit of the hCG could induce *in vitro* responses in α -subunit-specific T cell hybridomas, but to a reduced extent when the α - subunit was in conjunction with the β - subunit (8). By comparison, hybridomas specific for the β - subunit could mount a similar immune response to both the β -subunit and to the combined α - and β - subunits. Human follicle-stimulating hormone α - and β - subunits are identical and homologous to the hCG α - and β -subunits respectively. Comparison of the immune responses induced by these proteins showed that the three dimensional structure of a molecule could play a critical role in the processing pathway. However, in kinetic experiments, expression

of the hCG β - subunit appeared to be independent of the quaternary structure of the hCG molecule. That being the case, the conformation alterations within the combined $\alpha\beta$ molecule mainly influenced processing of the α -subunit rather than of the β -subunit (8).

One feature that can influence the quaternary structure of an antigen is the presence of disulphide bonds. Li *et al.* have shown that mutagenesis of cysteine residues may minimally disrupt the abundance and overall conformation of a protein, but that disruptions in the disulphide bonds influenced the MHC class II expression of dominant and subdominant T cell epitopes. These data suggest that disulphide bonds can regulate antigen processing both locally and at distant sites, influencing epitope selection within the MHC class II pathway (61).

1.5. – Processing of antigens for presentation by T cells

Antigens that bind APC receptors are internalised by receptor-mediated endocytosis and results in the antigen-receptor complex enclosed within endosomes. As the endosome progresses through the cell, the interior becomes increasingly acidic and it eventually fuses with lysosomes. These lysosomes contain acid proteases (for example, cathepsins D and E) that are activated at low pH and digest the antigen into peptide fragments (5, 62, 63). Later on in the process, vesicles containing MHC class II complexes fuse with the vesicles containing antigen-derived peptides.

1.5.1. – The mechanics of processing

MHC class II antigen complex assembly and presentation starts with a translocation of the MHC class II protein into the endoplasmic reticulum (ER). The ER is associated with relatively high concentrations of unfolded and partially folded peptides so the MHC class II molecules must be prevented from binding prematurely to other peptides, so in order to prevent aberrant peptide binding, the MHC class II binding groove is blocked by Ii. This protein forms trimers and each subunit binds covalently to the MHC class II α : β heterodimers. Ii binds to the MHC class II molecule in such a way that part of its polypeptide chain actually lies within the binding groove, preventing binding of peptides or partially folded proteins. Additionally, while the MHC class II complex is being formed, the component parts are associated with calnexin. When the proteins that make up the MHC class II molecule are in place, as well as the Ii, the calnexin is removed and the MHC class II: Ii complex is released from the ER. It has been shown that MHC class II molecules produced without Ii remain in the ER and are eventually destroyed in the same way as misfolded proteins (64-67).

As described earlier, processing mechanisms also have an important role in determining antigen presentation. The processing of antigen occurs within the lysosome and fragments are generated by enzymatic cleavage. The major enzymes involved in these processing steps are cathepsins D and E, and asparaginyl endopeptidase (AEP) (68, 69). The processing of TTD (55, 70) and of the non-collagenous domain of the Goodpasture antigen, the α 3 chain of type IV collagen (α 3(IV)NC1) (71) influences the processing of the complete protein. Both proteins require “unlocking” in order to be processed fully and for peptides to be presented on the surfaces of APCs .

In particular, processing has a major influence on TTD presentation. Several experiments have been undertaken using a specific region of TTD, the C fragment (TTCF). This fragment is responsible for binding to neurons via gangliosides (72, 73). This is important for the development of characteristic prolonged muscle spasms seen during the disease. It has been shown that processing of the TTCF fragment involves cleavage at asparagine residues 873, 1184 and 1219 by asparagine endopeptidase (68). Subsequent work has shown (by mutating the asparagine residues) that the mutant protein without asparagine at these sites is highly resistant to processing and that the asparagine at 1219 is obligatory for optimal presentation of many T cell epitopes within this protein (69). Cleavage at this point effectively “unlocks” the tetanus molecule for processing.

1.5.2. – The mechanics of class II binding

The Ii does also have a second important function: it directs the MHC class II molecules to a low pH endosomal compartment in order for peptide loading to occur. This compartment is known as the MIIC (MHC class II compartment) and occurs late in the endosomal pathway (74, 75). The MHC class II complexed with Ii remains in this compartment for up to four hours, during which time the Ii is cleaved by acid proteases in a very specific sequence of events (76). The initial cleavage events result in a truncated Ii and this ensures the molecule is retained in the proteolytic compartment (67). Subsequent cleavage events remove the membrane-associated fragment of Ii and leave a short fragment of the Ii (class II-associated invariant-chain peptide, or CLIP) in the peptide binding groove. No other peptide can bind the MHC class II molecule while the CLIP fragment remains associated

with the binding groove. CLIP must either dissociate or be displaced in order for peptide binding and translocation to the cell surface membrane to occur (67, 77).

In vitro experiments have shown that removal of CLIP and peptide loading can be directly catalysed by the HLA molecule, DM, suggesting the HLA-DM also has a role in the removal of CLIP from MHC class II molecules *in vivo* (64). Subsequent work has shown that HLA-DM and HLA-DR can be co-precipitated, implying association under steady state conditions *in vivo* (78). The association is favoured under low pH conditions, supporting the hypothesis that this association occurs during MHC class II peptide loading in the MIIC. It has been shown that the DR-DM association is transient and the DM also associates with MHC class II: peptide complexes (64). Additional work *in vitro* showed that HLA-DM has a degree of specificity for MHC class II molecules bound to CLIP, and is directly involved in peptide loading of the MHC class II molecules by preferentially binding CLIP and causing it to dissociate from the HLA molecule. These findings suggest a role for HLA-DM in prolonging the survival of empty class II molecules within the MIIC, a function likely to be important in peptide loading and presentation (79). More recent publications have concentrated on the nature of the HLA-DR/HLA-DM interaction, with Pashine *et al.* determining that the HLA-DM can alter the conformation of the MHC class II binding groove as well as edit the sequence of MHC class II-bound peptides (80). Mutating the HLA-DR molecule such that the HLA-DM binding site is acidic can disrupt the HLA-DM MHC class II interaction and the editing process. This group have also postulated that the HLA-DM molecule actually makes the HLA-DR molecule less specific (80). The most recent data describing the HLA-DR/HLA-DM interface has shown, through mutational analysis, that the DR-DM

interaction is inhibited by glycosylation of the HLA-DM molecule, supporting the need for a close interaction between the two molecules. The interface was described such that the presence of an acidic cluster of amino acids at the peptide N-terminus may be utilised as a possible dissociation mechanism (81). Additional work has suggested that HLA-DM is effectively a chaperone protein, and the inability of HLA-DM null mice and cell lines to remove the Ii fragment from the MHC class II molecules on the cell surface supports this idea (66, 80).

1.6. - Impact of MHC class II type on epitope processing and presentation

In 1979, Corradin *et al.* used enzymatic processing of cytochrome c to generate peptide fragments and then tested recall responses by T cells isolated from mice immunised with whole cytochrome c, in an attempt to determine specific T cell epitopes (82). This experimental design was then used to determine T cell epitopes in multiple sclerosis using myelin basic protein as the target antigen in a murine multiple sclerosis model, experimental autoimmune encephalomyelitis (83). Similar work was published using recombinant peptides to study epitopes in myasthenia gravis (84, 85), rheumatoid arthritis (86) and type 1 diabetes (87).

It was also noted that different mouse strains responded to different epitopes within the acetylcholine receptor, the target autoantigen in myasthenia gravis (88). This work concluded that epitopes specifically recognized by T lymphocytes of

patients with myasthenia gravis also represent specific T-cell epitopes in the autoreactivity to the acetylcholine receptor in mice (the autoreactive peptides described in humans were tested in inbred mice) and that immune responsiveness to these peptides is influenced by the genetic make-up of the responding mouse strains. This is corroborated by the knowledge that most autoimmune diseases are linked to specific HLA molecules in humans (89). In particular, Pette *et al.* published work investigating immune responses to myelin basic protein (linked with multiple sclerosis) with presentation of the peptides restricted to HLA DR2 (90). The work involving HLA-restriction in autoimmune model systems was assisted further with development of transgenic mice that expressed HLA molecules in lieu of the murine equivalents IAB and/ or IEB (91). These transgenic animals could be used to determine specific T cell epitopes that, when presented to reactive T cells, resulted in autoimmune reactions. Since then, transgenic mice have been used to identify possible T cell epitopes in many autoimmune diseases, including type I diabetes (92) and multiple sclerosis (93).

1.7. – Genetics and predisposition to autoimmunity

It is becoming more and more apparent that susceptibility to autoimmune diseases is influenced by complex genetic interactions in which multiple genes act in concert to contribute to a disease, yet have no or only modest effects independently. The major genetic factor predisposing towards autoimmunity is HLA type (94), but polymorphisms within a few common genes have been shown to contribute to

multiple autoimmune disorders (PTPN22 and IL-23 for example (95)). While currently only a few genes with influence are known, advances in genetic research are likely to rapidly expand the number of genes and specific genetic factors with an effect on overall predisposition. That being the case, it is currently unclear how common genetic variation compared with multiple rare variants will contribute to overall disease susceptibility (95). Even with genes displaying a marked association with autoimmunity, the likely mechanisms by which the genes contribute to risk remain unknown. These genes include intracellular signalling molecules, transcription factors, cytokines and cytokine receptors (including IL-2), and membrane receptors or relevant co-stimulatory molecules (95), while the autoimmune diseases include type 1 diabetes and Crohn's disease (96-98). This variety in gene loci suggests that signalling processes are very important in the overall development of autoimmunity, but that there are very disparate mechanisms that may subtly interact or work in complete independence.

One gene conferring increased susceptibility to autoimmunity is PTPN22, an intracellular tyrosine phosphatase. Single nucleotide polymorphisms (SNP) have functional correlates on the protein produced: a variant in which the arginine at amino acid position 620 is substituted with a tryptophan residue has been shown to change functional properties and confer a 2-fold increase in the likelihood of developing rheumatoid arthritis (99) or type 1 diabetes (100). This risk increases to 3-4-fold if the person is homozygous for the tryptophan-coding allele. In terms of strength of association, the tryptophan allele of PTPN22 is second in importance only to the MHC genotype for these two diseases (95). Other autoimmune diseases that have been linked to this genetic variant include Grave's disease (101),

Hashimoto thyroiditis (102), and myasthenia gravis (103). In addition, a murine line in which the mouse ortholog of PTPN22 (*Lyp* or *PEP*) is knocked out exhibits enhanced T cell activation combined with increased production of antibody (104).

Polymorphisms within cytokine and cytokine receptor genes influence autoimmune disease. The best described association between a cytokine receptor gene and autoimmunity is between Crohn's disease and the glutamine 381 variant of the IL-23 receptor (105-107). This association has also been shown in psoriasis and ankylosing spondylitis (108, 109). The IL-23 receptor shares a subunit with the IL-12 receptor, so autoimmune interactions and correlations between IL-23 are also inextricably linked to the IL-12 receptor. However, the SNP associated with the IL-23 receptor and Crohn's disease is actually on the unshared IL-23 receptor subunit. Amino acid 381, normally an arginine residue, is substituted with a glutamine residue and this substitution results in a strong preventative influence upon the development of Crohn's disease, although the functional significance of this change has not yet been elucidated (95).

The additional association of both Crohn's disease (106) and psoriasis (109) with the β subunit of the IL-12 cytokine itself adds to evidence that the balance in activity of IL-23 and IL-12 cytokine pathways is an important component of disease pathogenesis in these disorders. This association has been supported by findings in animal models, and recent data about the role of both IL-12 and IL-23 in differentiation of Th17 cells have also supported the evidence published from animal models (110). In particular, both IL-12 and IL-23 are upregulated in Crohn's disease

and trial therapies involving blockade of these cytokine pathways are showing promise for effective treatment of both Crohn's disease and psoriasis (96).

Polymorphism within the major T cell proliferative cytokine IL-2 has been linked with predisposition to coeliac disease (111). Polymorphisms within IL-2 have also been linked to type I diabetes, rheumatoid arthritis and Grave's disease (97, 105, 112), but less definitively. In particular, there are links between polymorphism in the IL-2 gene as a risk factor in non-obese diabetic mice (98), but no obvious functional variants have been identified in the non-obese diabetic mouse gene sequence (97). However, there are positive correlations between human type I diabetes and functional variation in the human IL-2 gene sequence (105, 113, 114).

The major genetic factor associated with predisposition to autoimmune disease is HLA type. Although numerous examples of specific HLA type and autoimmunity are documented, the specific mechanisms remain undefined. There are numerous known SNPs across the entire MHC class II loci, an area with massive genetic complexity, and it has been shown that genes outside the specific HLA genes, but within the MHC class II loci, may also influence autoimmunity (95). Multiple sclerosis and HLA-DR2 (of which HLA-DR15 is a subtype) (90), rheumatoid arthritis and HLA-DR4 (115), and Goodpasture's disease and HLA-DR15 (41, 116) are all examples of autoimmune diseases with known associations to a specific HLA genotype.

The mechanisms by which these HLA genotypes influence and predispose towards autoimmunity are not understood, but are likely to relate to the the particular capacity of the HLA molecule to present particular peptides. This association appears to correlate with features in the class II peptide binding groove (and by

inference the peptides best presented). These HLA genotypes may also have a higher affinity for antigen fragments that are not the major naturally processed peptides and are immunologically subdominant or cryptic (1, 50, 117-119). A current theory is that these subdominant peptides may not have been presented within the thymus during central tolerance. This suggests that T cells are able to recognise immunologically subdominant peptide fragments from self within the MHC class II peptide groove and become activated by the autoantigen. This could result in a proliferative response usually seen to exogenous antigen (120, 121). This in turn promotes an immune response to the self-antigen that is perpetuated by the gradual destruction of the tissue containing it. Since these peptides could be derived from slightly different processing mechanisms, effectively the T cells respond to different epitopes within the same protein (44, 45, 120, 122). In Goodpasture's disease, HLA-DR7 and HLA-DR1 have been shown to protect against the disease. In particular, the presence of HLA-DR7 ensures that despite the presence of DR15, the disease does not develop. The association between Goodpasture disease and HLA-DR15 is such that approximately 80% patients with Goodpasture's disease express this MHC class II molecule.

1.8. – Tolerance and the CD4 T cell response to self-antigen

In autoimmune disease, the immune system responds to an endogenous antigen, to self. A collection of mechanisms exists to prevent immune activation by self-antigen. This system is known as tolerance and is usually considered to comprise central and peripheral mechanisms. Central tolerance determines which T

cells are allowed to mature and to enter the periphery, while peripheral tolerance controls aberrant activation of the T cells once they are in the periphery.

1.8.1 – Central tolerance

How the body recognised self from non-self became a major point for discussion. With the discovery of mechanisms underlying how the immune system presents exogenous antigen, Owen first introduced the concept of tolerance in the immune response, in 1945. His experiments showed that monozygotic bovine twins had two types of erythrocyte circulating within their blood, but that they did not mount an immune response to the circulating “foreign” cells (123). Since then, the mechanisms underlying immunological tolerance have been studied in depth. The Nobel prize for medicine in 1960 was jointly awarded to Sir Frank Macfarlane Burnet and Peter Medawar for their work investigating acquired immunological tolerance with particular relevance to transplantation (124-128).

Of particular interest for this project was work by Lo and Sprent, in 1986 (129), demonstrating the importance of the thymus in developing T cell tolerance. In these experiments, mice were thymectomised then engrafted with a replacement thymus from a 14 day old foetus. The donor thymus was either the same genotype as the original mouse, or from a different strain of mouse so of a different genotype. These mice were then irradiated to remove the circulating T cell population, and given bone marrow intravenously to provide stem cells. After sufficient time had passed to enable the immune system to recover following the irradiation, the animals were primed with antigen (keyhole limpet haemocyanin, or KLH), and the proliferative responses of the T cell in the various strains were analysed. These experiments showed that mice with an engrafted thymus had a greater immune

response to the antigen determinants favoured by the engrafted thymus genotype, so showed that the thymic environment is necessary for T cells to develop MHC class II recognition. Additional experiments in which the foetal thymuses were incubated with deoxyguanosine prior to graft (deoxyguanosine destroys intrathymic cells of macrophage and dendritic lineage, so all APCs), showed that the bone-marrow derived cells were not necessary for the development of MHC class II recognition by T cells (129).

Central tolerance has since been defined as the process of refining the T cell repertoire by testing for the T cells that recognise MHC-peptide complexes too weakly to be useful or too strongly to avoid autoimmunity. Tolerance begins, as described by the experiment above, in the thymus. Double-negative T cells (express neither CD4 nor CD8 on their surfaces) emerge from the bone marrow and migrate to the thymus where they begin to mature and express both CD4 and CD8, becoming double-positive T cells. These then further mature into single positive T cells depending on their affinity for either MHC class I or MHC class II, and start being selected according to their ability to bind MHC-peptide complexes.

1.8.2. – The role of the thymus in determining tolerance

Antigen recognition is crucial for the development of the T cell repertoire. The actual T cell selection is very much compartmentalised within the thymus. The thymus consists of lobes, each of which is organised into outer cortical and inner medullary regions, each containing specialised cells. Rapidly proliferating immature cells are associated with the outer cortex; as cells enter the inner cortex they are expressing both CD4 and CD8 and are probably being tested for their ability to bind MHC class II (130, 131). The medulla contains the mature, CD4- or CD8-positive

lymphocytes, medullary epithelial cells, bone marrow-derived macrophages and dendritic cells. It is not currently known whether the expression and concentration of MHC class II on antigen presenting cells and their isolation have an influence on the selection processes. It is currently thought that thymic endothelial cells express MHC class II molecules on their surfaces, T cells that can bind the MHC class II molecules are kept, while those that do not recognise the MHC class II molecules are deleted (131). This leaves a population of cells that recognise MHC class II. Thymic cells within the corticomedullary junction of the thymus and the thymic medulla have been shown to express MHC class II molecules containing self-derived peptides in their binding groove (131). Under these conditions, all T cells binding with too high an avidity for the MHC class II: peptide complexes are more likely to be deleted since these are the cells likely to induce autoimmunity (negative selection). This could leave a T cell population that is capable of recognising MHC class II: peptide complexes, but without a high enough avidity to cause aberrant autoimmune reactions. It could be assumed that these cells therefore only present peptides from processed endogenous antigen and not antigen from the circulation. It has been shown that peptides derived from the Goodpasture antigen, the $\alpha 3$ chain of the non-collagenous domain of type IV collagen ($\alpha 3(\text{IV})\text{NC1}$) (4) and peptides derived from insulin (132) are expressed in the thymus. This suggests that there is potential opportunity for negative selection of these antigens, and therefore supports the idea of centralised tolerance to antigens with which the circulating T cells may come into contact while in the periphery. T cells that are released into the periphery, the mature T cell pool, may recognise MHC class II molecules associated with a foreign peptide, or a peptide not expressed by the thymus, but some that respond to

self-antigen within the binding groove escape central deletion (133). Peripheral tolerance controls them.

Experiments with mice that express MHC class II molecules without peptide on their cortical epithelial cells show a normal level of positive selection for ability of their T cells to bind MHC class II, but impaired selection for ability to bind MHC class II: peptide complexes, supporting the hypothesis that these cells play a role in selecting T cells for ability to bind MHC class II molecules. It has also been found that bone marrow-derived macrophages and dendritic cells are responsible for the removal of at least 50% of all T cells positive for MHC class II binding ability (130).

1.8.3. – The role of autoantigen in tolerance

Antigens targeted in autoimmune diseases have been shown to be presented within the thymus and are therefore hypothesised to induce central tolerance. Tolerance can prevent those autoimmune diseases in the majority of the population (diabetes, multiple sclerosis). A possible explanation for the occurrence of autoimmunity lies in peptide presentation during T cell selection, so within the development of tolerance. Although the thymic epithelial cells are presenting peptides from various endogenous antigens, the peptides have to be processed and exposed to MHC class II molecules in the same way as exogenous antigen. This suggests that during the development of the T cell repertoire, that epitopes presented within the thymus are major naturally processed peptides. It could therefore be hypothesised that during development of autoimmunity, the processing differs slightly so the peptides presented within the MHC class II binding grooves on APCs are different from those normally presented and to which the T cells were exposed in the thymus. For tissue-specific antigens like $\alpha 3(\text{IV})\text{NC1}$, this is not always thought

to be the case as these antigens may have some protection from immune responses through sequestering the whole antigen (134).

While it is now accepted that central tolerance and T cell selection occurs in the thymus, there are questions arising regarding how the thymus could actually present all possible self-antigens. The thymus cannot and does not present all possible antigens, but there is evidence to support expression of antigens such as insulin (diabetes) (132), proteolipid protein from the brain (experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis) (135), and fragments of type IV collagen (Goodpasture's disease) (4). This information suggests that cells within the thymus can take advantage of so-called promiscuous gene expression (136), so are a unique cell type capable of expressing a diverse range of tissue-specific antigens. It has been shown that this ability is maintained through thymic T cell output and regulated by the nuclear protein autoimmune regulator (AIRE) (137-139). It was hypothesised that this capability may enable presentation of the limited range of self-antigens that would otherwise be temporally or spatially secluded from the immune system. However, upon testing, the actual presentation of the self-antigens within the thymus appeared more random and was not confined to sequestered self-antigens (136).

1.8.4. – Peripheral tolerance

Peripheral tolerance can be described as the mechanisms by which the circulating T cell population is prevented from reacting to self. The mechanisms resulting in regulation of peripheral T cell activation can be diverse, including limiting access to tissues, induction of apoptosis for aberrantly activated T cells and directly regulating effector T cell responses by other T cells.

While some tissues and antigens are sequestered (immunologically privileged), some tissues are frequently exposed to the immune system. Certainly, the antigens that are sequestered are unlikely to activate T cells, through inaccessibility to the immune system (134). Privileged sites initiate mechanisms that cause activated T cells to either undergo apoptosis or encourage them to migrate away from the area. For example, immune privilege has been investigated in respect of T cell access to the retina. D'Orazio *et al.* showed that local cytokine release (TGF- β , IL-10) impedes the entry of T cells in the retina (140), while Sonoda *et al.* showed that cytokines and Tregs prevent self-deleterious responses of T cells that reach the retina (141). Tissues transplanted into immune privileged areas have increased chances of survival and reduced rejection, for example corneal allografts (142), while stem cells that have potential uses in transplantation have also been shown to exhibit some immune privilege (143).

A balance of co-stimulatory signals affects immune homeostasis and self-tolerance. Naïve T cells require two signals to proliferate and differentiate. The first is triggered by TCR recognition of the appropriate MHC class II: peptide complex, while the second signal is from CD80 and CD86 co-stimulatory molecules on the APC surface. Depending on the co-receptor used, these signals can be interpreted in different ways. Some examples of different interpretations through use of different co-receptors include CD28, a molecule constitutively expressed on T cell surfaces that enhances cell survival (144), CD40 ligand expression that prevents T cell anergy (145), while ligation of CTLA-4 (CD152) inhibits T cell responses (146).

Both naturally occurring and antigen-induced Treg cells have a major influence on peripheral tolerance and maintaining immunological homeostasis by ensuring

overwhelming immune responses to antigen or autoantigen are unable to proceed. Overall, Treg cells suppress immune responses. They suppress activation of dendritic cells, Th1, Th2, Th9 and Th17 cells, mast cells, basophils and eosinophils. They suppress effector T cell migration and IgE production in responses to allergens (17). One mechanism by which suppression is induced is by rendering the circulating T cells anergic. This means that although the T cells are able to be activated, they require higher levels of stimulation in order to differentiate into the appropriate effector T cells. Treg cells can also result in deletion, either by inducing apoptosis, or by neglect, although nTregs use cell-cell contact (25) and iTregs use cytokine secretion (26) to achieve similar results. In order to maintain a healthy circulating T cell population, the peripheral T cells need access to certain cytokines to keep them alive but inactive, in stasis. In order for a cell to be deleted by neglect, the cell fails to receive costimulatory signals through CD28. Tolerance in the context of lack of CD28 costimulation can result from Fas-independent peripheral deletion (147).

Suppression of antigen-specific immune responses by IL-10, a known suppressive cytokine of T cell proliferation and cytokine production, is thought to be important in ensuring peripheral tolerance to allergens, autoantigens, transplantation antigens, and tumour antigens (148). In mice, it has been shown that IL-10 administration prior to allergen treatment induces antigen-specific T cell unresponsiveness and demonstrates the pivotal role of IL-10 in the establishment of peripheral T cell tolerance (149). Inhibition of graft-versus-host disease by IL-10 and allograft rejection in patients with severe combined immunodeficiency undergoing HLA-mismatched bone marrow transplantation provide additional

evidence for a key role of this cytokine in the induction and maintenance of peripheral tolerance (150). The mechanism by which IL-10 suppresses T cells involves that direct blocking of inducible costimulator, CD2 and CD28 costimulatory signals in a rapid signal transduction cascade and occurs through direct use of the Src homology 2 domain-containing protein tyrosine phosphatase (151) by IL-10 (151, 152). This ensures that the T cells cannot become activated.

Overall, peripheral tolerance maintains immunological reactivity in the periphery, while preventing aberrant responses that could result in an autoimmune reaction.

1.9. - The scope of tolerance across peptides of an antigen

The scope of tolerance across peptides from an antigen has been shown using mice that are transgenic for expression of hen egg lysozyme (HEL) (120). In particular, work by Gapin *et al.* has shown that tolerance is not only induced to the immunodominant peptide of HEL, testing immune responses of HEL transgenic mice, but also to subdominant or cryptic peptides within HEL (153). This work was further expanded to show that while dendritic cells mainly present the immunodominant peptide, B cells are capable of diversifying the T cell response by presenting a more heterogeneous set of peptide-MHC complexes, and that a peptide designated subdominant due to inefficient processing and reduced immunogenicity, was equally tolerogenic as the immunodominant peptide (154). This work is corroborated by the experiments carried out by Peterson *et al.*, who found that there is equal tolerance among epitopes of HEL expressed at different levels on APCs, and

that central tolerance to a limited number of peptide-MHC complexes occurs with high sensitivity (155). This was also shown by Gammon and Sercarz, who showed that mice were tolerant to HEL despite the presence of HEL-reactive T cells. They proposed that these HEL-reactive T cells escape tolerance because some minor determinants are only available in relatively low amounts after *in vivo* processing. As a result, these T cells are not normally activated but can be stimulated under special circumstances and circumvent tolerance (156).

The current consensus is that tolerance to HEL can be initiated in transgenic mice, even with restricted distribution of the HEL throughout the animal, as determined by Ham *et al.* They generated transgenic mice that expressed HEL under the control of the rhodopsin promoter and found that these mice were resistant to the expected ocular inflammation following immunisation with HEL. They then found HEL mRNA in the thymi of transgenic animals and, following cross-breeding with a transgenic mouse that expresses HEL specific T cell receptors, found that there were reduced numbers of HEL-specific T cells in the circulation of these animals (157).

1.9.1. – Tolerance across peptides in autoantigens

Importantly, tolerance has also been shown in mouse models of experimental autoimmune disease. Fairchild *et al.* showed that the main autoimmune response in experimental allergic encephalomyelitis (EAE) was directed towards the subdominant epitopes of myelin basic protein, rather than towards those peptides displaying immunodominance (121). They found that a modified subdominant epitope was expressed on the APC surface for almost four hours, while the wild-type epitope bound very transiently and could not be measured on the APC surfaces. These data suggested that epitopes could escape tolerance mechanisms by binding

transiently without enabling autoreactive T cells to bind and be deleted during negative selection (121). This work was corroborated by Joosten *et al.* who investigated specific T cell responses in the Lewis rat to autoimmune epitopes from different disease models including EAE (myelin basic protein), autoimmune arthritis (heat-shock protein 65) and myasthenia gravis (acetylcholine receptor). They found that autoimmune epitopes were intermediate to poor binders of the MHC class II molecule compared to other, known immunodominant peptides (158). They also found that single amino acid substitutions at defined positions were sufficient to turn certain peptide into good binders, and postulated that these data could be relevant to the design of competitive binding peptides in the treatment of autoimmunity (158).

More recent work by Kanagawa *et al.* found that specific MHC class II genotypes had increased numbers of autoreactive T cells. This work showed that non-obese diabetic (NOD) mice that expressed I-A^{g7} molecules on their cell surface contain a high number of autoreactive T cells compared to NOD mice with I-A^b. It was subsequently found that the autoreactive T cells have a high level of reactivity to APCs not pulsed with antigen, so the T cell repertoire is skewed towards autoimmunity, possibly due to weak binding properties of the I-A^{g7} (159). The situation with these NOD mice is somewhat akin to that with mice bearing I-A^u. If these I-A^u mice are immunised with an EAE epitope, EAE develops despite a very weak binding interaction between the peptide and the I-A^u molecule (159).

Additional work on EAE models has shown that encephalitogenic T cells that escape tolerance either recognise short-lived peptide/ MHC complexes, or express TCRs with unique specificities for stable complexes. It was hypothesised that a decrease in the level of expression of myelin basic protein epitopes on cells that

mediate tolerance would enable more T cells to escape tolerance. This decrease would therefore result in a greater percentage of the repertoire consisting of T cells that would require a lower level of antigenic exposure for activation, and may have implications for the development of autoimmunity, or multiple sclerosis in particular. This work also implies that genetic or environmental influences that decrease the efficiency of induction of tolerance may change the repertoire of self-reactive T cells to include a greater number of T cells with the potential to be triggered by lower concentrations of self-antigens. This suggests that both subdominant and immunodominant peptides may be targets in the initiation of autoimmune disease (160).

Additional work has suggested that immunisation of HEL transgenic animals with exogenous HEL in conjunction with CpG oligonucleotides can break tolerance to HEL, as measured by high titres of anti-HEL antibodies, but does not induce autoimmune disease. The animals used, even with long term immunisation schedules, did not develop T cell responses to endogenous antigen or tissue damage, despite the presence of HEL-specific antibodies. This suggests that B cell tolerance is broken by this system, rather than central T cell tolerance, but work is ongoing to clarify this point (161).

Another explanation for lack of tolerance to specific epitopes from autoantigens is that of destructive processing. This theory suggests that some potential T cell epitopes may contain protease cleavages sites and are therefore destroyed during normal processing mechanisms (55). With self-proteins, this may have more serious consequences if other factors can modulate the epitope crypticity. There is also evidence to suggest that antigens could be subjected to processing in

such a way that premature lysosomal proteolysis is prevented, resulting in modulation of peptide binding and the potential for enhanced MHC class II- peptide complex formation (67). One such example is a peptide from myelin basic protein (MBP 85-99). In the case of MBP 85-99, there is a known inverse correlation between AEP activity and peptide concentration but mechanisms causing the peptide to become cryptic and presented by APCs are currently unknown (55).

1.10. – Goodpasture's disease and the Goodpasture antigen

Goodpasture's disease is a relatively rare autoimmune disease causing renal failure and pulmonary haemorrhage. There are 1-2 cases per million people per year and, despite the scarcity of new cases, this disease has been well characterised (162). There is a strong association between Goodpasture's disease and the HLA-DR15 molecule (approximately 80% of patients carry this HLA molecule), and the autoantigen has been well defined and major naturally processed antigen derived peptides have been identified. The Goodpasture antigen was cloned in 1992 by Turner *et al.* (163), and since then the crystal structure of the $\alpha 1(\text{IV})\text{NC1}$ chain has been determined (164), enabling some elucidation of the potential crystal structure for the $\alpha 3(\text{IV})\text{NC1}$ domain. This means that Goodpasture's disease had potential for use as a model system to analyse the interactions between the antigen and the HLA-DR15 molecule. In particular, models of this disease have the potential to be used to clarify whether HLA-DR15 enhances the autoimmune response to $\alpha 3(\text{IV})\text{NC1}$ peptides by efficient presentation to the autoreactive T cells, or whether there is

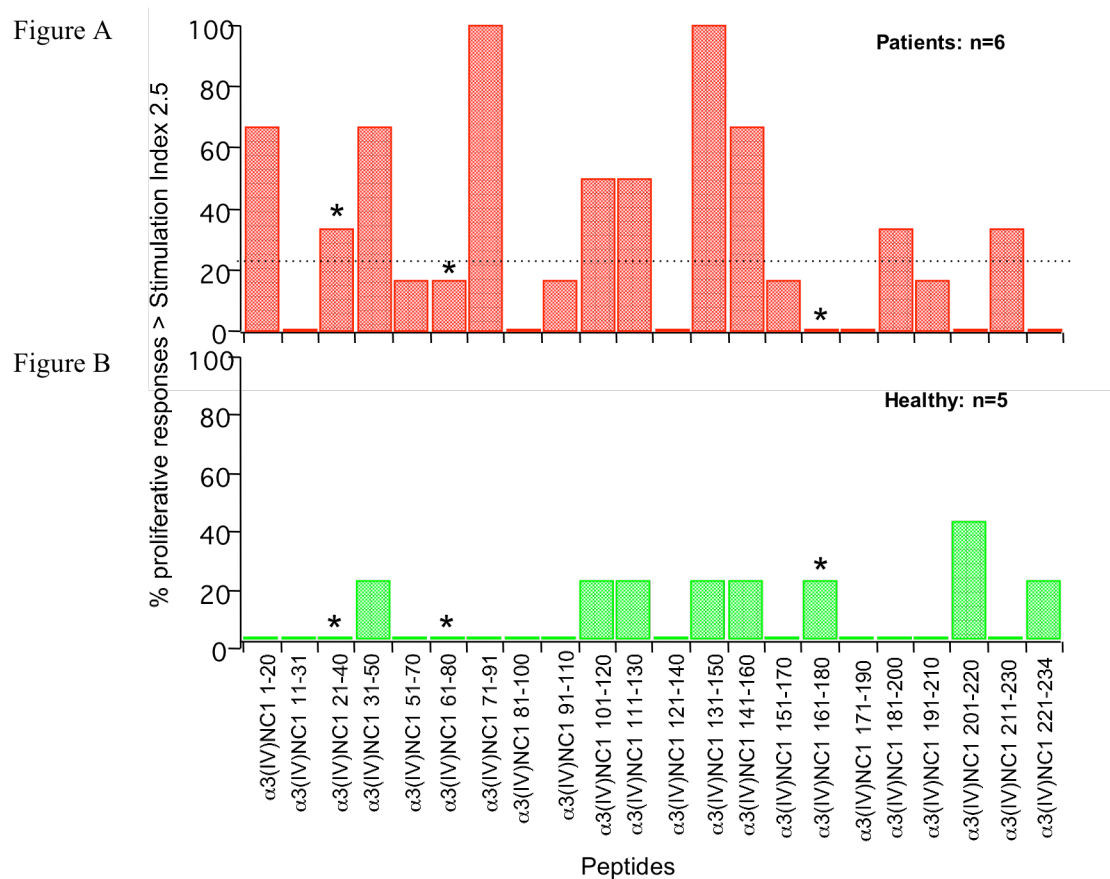
failure to induce tolerance within the thymus by poor presentation of $\alpha 3(\text{IV})\text{NC1}$ peptides in the thymus (unlikely as $\alpha 3(\text{IV})\text{NC1}$ has been shown to be presented in the human thymus (4)). It could also be used to clarify the role of so-called protective class II molecules in deletion of autoreactive T cells or sequestering $\alpha 3(\text{IV})\text{NC1}$ derived peptides (116).

Research has shown that the presence of HLA-DR15 is unlikely to increase susceptibility to Goodpasture's disease by presenting a peptide particularly well since, in general, $\alpha 3(\text{IV})\text{NC1}$ derived peptides bound HLA-DR15 with similarly high affinity as they would HLA-DR7. Only some of these peptides evoked patient T cell responses (peptides containing amino acid residues 71-90 and 131-150) and these were not the peptides found to be most abundantly presented on APC surfaces (peptides containing amino acid residues 21-40, 61-80 and 161-180) (41). It was also found that HLA-DR7 or HLA-DR1, so-called protective haplotypes, on average were able to present particular $\alpha 3(\text{IV})\text{NC1}$ peptides better than HLA-DR15, although HLA-DR15 did present some peptides better than HLA-DR7. It has been suggested that HLA-DR7/1 could protect by capturing peptide and preventing their display bound to HLA-DR15. Binding data obtained has indicated that biochemically detectable peptides isolated from HLA-DR15 homozygous APCs would preferentially bind HLA-DR7/1 binding grooves in HLA-DR15, HLA-DR7/1 heterozygous APCs. This implies that the protective effect of HLA-DR7/1 molecules may be due to increased affinity for the peptides to which HLA-DR15 binds in order to cause disease (41).

1.10.1. – Goodpasture antigen and processing

Figure 1.4. – T cell immune responses to overlapping $\alpha 3(IV)NC1$ peptides in Goodpasture patients and HLA-matched control subjects.

These charts show the percentage of the individuals tested whose peripheral blood mononuclear cells proliferated (Stimulation Index >2.5) to the indicated $\alpha 3$ peptides. Immune responses to the overlapping 20mer peptides are shown from Goodpasture patients (figure A) and healthy, HLA-matched control subjects (figure B). The major naturally processed peptides, determined by elution studies are marked *, do not elicit the greatest immune responses in either control subjects or in Goodpasture patients, suggesting that tolerance to the best presented peptides is secure in health and in Goodpasture's disease. (Chart adapted from Cairns *et al.* (1)).



In order to present peptides derived from the $\alpha 3(\text{IV})\text{NC1}$ protein, specific cleavage events at certain points in the protein must occur in order to enable complete processing. The Goodpasture autoantigen, requires a specific cleavage event that subsequently enables the rest of the molecule to be processed and presented. Studies by Zou *et al.* determined that processing by cathepsin D/E was essential for the unlocking of the protein as the processing could be completely abrogated by pepstatin A, a specific cathepsin D/E inhibitor. They also found that purified cathepsin D generated the same major $\alpha 3(\text{IV})\text{NC1}$ fragments as entire lysosomes, suggesting that the cathepsin D cleavage events are involved in $\alpha 3(\text{IV})\text{NC1}$ processing (71). The differences between processing and T cell responses is illustrated by figure 1.4. in which immune responses to overlapping 20mers derived from the Goodpasture antigen, $\alpha 3(\text{IV})\text{NC1}$, were compared between Goodpasture patients and healthy HLA matched controls. The figure shows that the major naturally processed peptides, as determined by *in vitro* elution studies ($\alpha 3(\text{IV})\text{NC1}$ 21-40, 61-80 and 161-180, marked *) are not the peptides that induced the greatest immune responses in 100% of the Goodpasture patients ($\alpha 3(\text{IV})\text{NC1}$ 71-91 and 131-150) (1). It has also been shown that the $\alpha 3(\text{IV})\text{NC1}$ protein, when processed and presented on the cell surface in conjunction with the HLA-DR15, is presented as two sets of three to five peptides centred on a common core sequence (nested sets) and that synthetic peptides within those sets bind the HLA-DR15 with intermediate affinity with IC_{50} values ranging from 1.1-6 μM (165). These data imply that the peptides processed and presented have a major influence on tolerance.

1.10.2. – Immunoglobulins and epitopes on the Goodpasture antigen

With the Goodpasture antigen being cloned (163) and the crystal structure determined (164), there have been increasing efforts to define the epitopes within the $\alpha 3(\text{IV})\text{NC1}$ protein that cause the autoimmune responses seen in Goodpasture's disease. In particular, work has concentrated on determining which specific epitopes are targeted by antibodies and cause pathology. Experiments by Prof. D. B. Borza's and Prof. B. G. Hudson's groups have shed light on various epitopes within the Goodpasture antigen. In particular, it was shown that antibodies from Goodpasture patients could bind to specific epitopes – E_A and E_B . E_A corresponds to amino acid residues 17-31, while E_B corresponds to amino acid residues 127-141 of the Goodpasture antigen (166). The antibodies from three Goodpasture patients were classified as reacting to either epitope, both epitopes or neither epitope (117). Of the two epitopes, E_A was found to be consistently immunodominant with antibodies to this epitope accounting for up to 65% of reactivity to the $\alpha 3(\text{IV})\text{NC1}$ molecule. This implies that the E_A epitope may have a role in pathogenesis since the T cell responses are seen mainly in response to this epitope. It has also been shown that these antibodies are capable of destroying the intact hexameric structure of the type IV collagen non-collagenous domains. This destructive binding could be a mechanism by which the disease pathogenesis is propagated (117). This binding is also interesting because the epitopes themselves are sequestered within the planes of the $\alpha 3(\text{IV})\text{NC1}$ that interact with the other α chains forming the overall hexamer (117, 167, 168).

The differences seen between epitopes for antibodies and epitopes for T cells are not unexpected as B cells recognise overall conformation and protein quaternary structure, while T cells fragments of the amino acid sequence. The epitopes to which

there is known antibody binding are not the epitopes to which there is a marked T cell response in humans (antibodies bind residues 17-31 and 127-141, whereas T cell responses are seen to amino acids 71-91 and 131-150). These are also not the major naturally processed peptides generated from processing of the $\alpha 3(\text{IV})\text{NC1}$ domain (residues 21-40, 61-80 and 161-180), although there is some sequence overlap between one of the major naturally processed peptides (21-40) and the antibody binding epitope, E_A – 17-31. This indirectly supports the idea that central tolerance is induced to immunodominant and major naturally processed peptides, such that immunosubdominant peptides become the peptide that induce a T cell response (120).

The work within my supervisors laboratory has mainly focussed on processing and T cell responses to $\alpha 3(\text{IV})\text{NC1}$, since processing is essential for T cell recognition to occur. In particular, Dr J. Zou determined the sequence of processing events for unlocking the protein through specific enzymatic cleavage reactions and time frames within which these occur (71). This was done by utilising lysosomal extracts and specific enzyme inhibitors to identify that cathepsin D is essential for the unlocking process. In addition, work from this group has shown that $\alpha 3(\text{IV})\text{NC1}$ is expressed in the thymus by thymic epithelial cells. This suggests that fragments of $\alpha 3(\text{IV})\text{NC1}$ may be presented to T cells during central tolerance, but that has yet to be verified (4). Despite this finding, it has been shown that healthy human blood contains T cells activated by fragment of the Goodpasture antigen (3). This supports the hypothesis that the T cells in a periphery are reactive to subdominant peptides against which central tolerance is not effective (120). My project aims to determine fragments of the Goodpasture antigen to which a T cell response is generated without

central tolerance, expanding our knowledge regarding the impact of tolerance on processing and the way in which T cells react.

1.11. – Summary and objective of thesis

To summarise, immunodominance is determined by, at best, multiple factors including affinity and processing. In Goodpasture's disease, the immunodominant peptides presented by the HLA-DR15 molecule are not the most efficiently presented, and peripheral blood mononuclear cells from Goodpasture patients show no immune responses to the major naturally processed peptides as determined by elution studies. A possible explanation for this is that central tolerance is to the most efficiently presented peptides and not those peptides with high affinity for the HLA molecule but that are less well presented. This also questions the scope of central tolerance to the renal autoantigen α 3(IV)NC1.

Some data published to date has indicated that the epitopes that induce autoimmune disease are not major naturally processed peptides, and that both processing and tolerance have roles in the development of autoimmunity (1, 153, 165). The bulk of these data was obtained from investigating Goodpasture's disease (1, 153, 165), but there has also been some work investigating thymic presentation of major naturally processed peptides in lupus (169) and diabetes (170). The way in which peptide processing, affinity for HLA molecules and tolerance interact is unclear. This study investigates T cell proliferative responses to specific peptides in the presence and absence of endogenous expression, therefore influencing central tolerance.

Potential epitopes can currently be examined by a combination of computer modelling and recall response assays. The computational approach is extensively utilised in vaccine research, to determine potential targets on rapidly evolving micro-organisms or viruses (171, 172). In addition, antibodies obtained from patients with autoimmune disease can be characterised and epitope specificity determined (173). The combination of computational analysis through predictive algorithms and biochemical binding analyses can be used to identify potential epitopes. *Ex vivo* cellular analysis through use of synthetic peptides and antigen fragments can then assess the accuracy of the computational and biochemical identification techniques. In combination, the methods can be powerful tools for the prediction of antigen epitopes, but one method should not be used at the exclusion of the others.

One of the aims of this project was to investigate the effect of tolerance on the immune response in animals. In particular, epitopes recognised within the Goodpasture antigen, $\alpha 3(\text{IV})\text{NC1}$, in the presence and absence of tolerance, were studied. These data could be compared with existing biochemical data. The data generated during this project could then enable further investigations into how central tolerance mechanisms influence the development of autoimmune diseases. While this project concentrates on the well-characterised Goodpasture antigen, the underlying tolerance mechanisms are not exclusive to this disease. The conclusions drawn during this project could therefore be applicable to other autoimmune diseases, and could aid development of successful treatment for autoimmune conditions.

My working hypothesis was that the specificity of autoreactive T cells is diverted from major naturally processed peptides because of tolerance mechanisms.

This predicts that if tolerance was removed, the immune response would focus on major naturally processed peptides as it would for exogenous antigen. It also suggests that, in the absence of tolerance, immune responses could be skewed in favour of specific peptide fragments by use of a B cell specific adjuvant.

The project plan was therefore to:

1 – Examine presentation of specific epitopes by developing a new antigen delivery system. This system could enable elements in the overall influence of antigen structure on immunogenicity to be distinguished, and isolate those elements that are due to effects on antigen uptake or on antigen processing (Chapter 3).

2 – Generate transgenic mice which expressed HLA-DR15 and could be KO or WT for $\alpha 3(\text{IV})\text{NC1}$ (Chapter 4);

3 – Prepare as far as possible ‘natural’ conformation antigen (Chapter 5);

4 – Immunise HLA-DR15 expressing $\alpha 3(\text{IV})\text{NC1}$ WT mice and determine the various T cell specific immune responses (Chapter 6);

5 – Immunise HLA-DR15 expressing $\alpha 3(\text{IV})\text{NC1}$ KO mice, determine immune responses and to generate hybridomas for future analysis of peptides, epitopes and the $\alpha 3(\text{IV})\text{NC1}$ antigen (Chapter 7);

Chapter 2 – Materials and Methods

2.1. – Materials

2.1.1. – General Chemicals and Reagents

Chemicals and reagents were obtained as follows: human immunoglobulin G (IgG) was provided by the Scottish National Blood Transfusion Service (Glasgow, Scotland). Coomassie stain and prestained protein marker ladders were from Fermentas (Glen Burnie, Maryland, USA). Acetic acid, ethanol, ethidium bromide, hydrochloric acid, histoclear, imidazole, isopropanol, nickel chloride, paraformaldehyde, sodium acetate, sodium dodecylsulphate and sodium hydroxide were from Thermo Fisher Scientific (Loughborough, UK). All murine genotyping primers were produced by GATC biotech (Konstanz, Germany) and were subjected to BLAST searches to ensure minimal cross-reactivity. Protein L primers and corrective adaptors were produced by VH bio Ltd (Gateshead, UK). Nitrocellulose, PuReTaq Ready-To-Go PCR beads and tritiated thymidine were from GE Healthcare (Buckinghamshire, UK). Lipofectamine was from Invitrogen (Paisley, UK). Dried milk powder was from Morrison's supermarket (Bradford, UK). NovaBlue singles, DH5 and BL21 *E. coli* were from Novagen (Merckbiosciences, Darmstadt, Germany). DNase and all restriction enzymes were from Promega (Madison, Wisconsin, USA). Midiprep kits and proteinase K were from Qiagen (Crawley, UK). Phenol was from Thistle Scientific (Glasgow, UK). Acrylamide, agar,

agarose, ampicillin, borate, bovine serum albumin (BSA), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), calcium acetate, calcium chloride, casein, collagenase (type VII), cyanogen bromide-activated sepharose beads, deoxycholic acid, 4',6-diamidino-2-phenylindole (DAPI), dimethylsulphoxide (DMSO), dithiothreitol (DTT), eosin, ethylenediamine tetraacetic acid (EDTA), fluorescein isothiocyanate (FITC), Freund's adjuvant (both complete and incomplete), glucose, glycerol, glycine, guanidine, haematoxylin, hen egg lysozyme (HEL), hexamine cobalt chloride, iodoacetic acid-linked sepharose beads (IAA beads), isopropyl β -D-1-thiogalactopyranoside (IPTG), Luria-Bertani (LB) broth powder, magnesium chloride (MgCl_2), magnesium sulphate, manganese chloride, morpholinoethanesulphonic acid, nitro blue tetrazolium (NBT), ovalbumin, phosphate buffered saline tablets, polyethylene glycol (PEG), potassium acetate, potassium chloride, sepharose beads, sodium chloride (NaCl), sodium hydrogen carbonate, Tris, Triton X100, Tween-20, urea and yeast extract were obtained from Sigma-Aldrich (St Louis, Missouri, USA).

2.1.2. - Tissue Culture reagents

Tissue culture plates and dishes were purchased from Corning Life Sciences (Lowell, Massachusetts, USA). Mouse IL-2 In vivo Capture assay kits, 40 μm cell strainers, 15ml and 50ml conical tubes were purchased from BD Biosciences (San Jose, California, USA). Tissue culture reagents Dulbecco's Modified Eagle's Medium (DMEM), RPMI, Hanks' Balanced Salt Solution (HBSS), Dulbecco's Phosphate Buffered Saline (PBS), Heat-Inactivated Foetal Bovine Serum (HI-FBS), penicillin/streptomycin, geneticin and L-glutamine (200mM) were purchased from PAA (The Cell Culture Company) (Pasching, Austria). Beta-mercaptoethanol (βMe),

Hypoxanthine- Aminopterin- Thymidine (HAT) and Hypoxanthine- Thymidine (HT) supplements were from Sigma-Aldrich (St Louis, Missouri, USA).

Recombinant mouse interleukin (IL)-2 was purchased from Sigma-Aldrich (St Louis, Missouri, USA). The cytokine was reconstituted as indicated in the datasheet and stored in aliquots at -80°C. Concalavalin A (ConA) was purchased from Sigma-Aldrich (St Louis, Missouri, USA), reconstituted as indicated in the datasheet and stored in aliquots at -20°C. Peptides AS345, AS346 and $\alpha 3(\text{IV})\text{NC1}$ 15mer peptides were synthetic and sequenced as described in appendix I, arrived lyophilised and were redissolved at 10mg/ml in DMSO before storing at -20°C. These peptides were HPLC purified to 90%.

2.1.3. – Animals

Generation of mouse class II knock out, HLA DR15-expressing, $\alpha 3(\text{IV})$ (COLIVA3) knock out or wild type is described in Chapter 3. $\alpha 3(\text{IV})$ knockout mice were obtained from Dr J Miner in St Louis, Missouri, USA (174). Mouse class II knock out, HLA-DR15 expressing mice were obtained from Prof D Altmann in London, UK (175). Prof Altmann's group inserted the HLA DR15 double transgene into the mouse class II knock out line generated by Cosgrove *et al.* (176) in 1991. C57Bl/6 mice used as controls were bred on site. All procedures were undertaken with approved licence from the Animal Scientific Procedure Division of the Home Office.

2.1.4. – Cell lines

BW5147 cells – T cell myeloma line used for fusion reactions to produce T cell hybridomas (ATCC, LGC standards, Teddington, UK).

CRC013 cells – Epstein-Barr Virus transformed B cells, transformed to express HLA-DR15 by Prof. D. Crawford's group, University of Edinburgh.

HEK293 cells – Human embryonic kidney cells, used for transfection reactions (ATCC, LGC standards, Teddington, UK).

2.1.5. – Antibodies

Antibodies were obtained from the following sources: mouse anti- human CD19-phycoerythrin (PE) and IgG₂-PE isotype were from Caltag (Buckingham, UK). Mouse anti-HSVtag was obtained from Novagen (Merckbiosciences, Darmstadt, Germany). Anti-Igk-fluorescein isothiocyanate (FITC), anti- Ig λ -FITC, anti-HLA DR-FITC, anti-mouse class II and IgG₂-FITC isotype were from AbD Serotec (Oxford, UK). Anti-mouse IgG FITC and anti-human IgG Alkaline phosphatase (Alk phos) were from Sigma-Aldrich (St Louis, Missouri, USA).

2.1.6. – Plasmid vectors

Restriction maps for all plasmid vectors are shown in Appendix II. Plasmids used were the pET25b expression vector (Novagen, Merckbiosciences, Darmstadt, Germany), pGEM-T cloning vector (Promega, Madison, Wisconsin, USA), pCR-BLUNT TOPO blunt cloning vector (Invitrogen, Paisley, UK) and pFLAG-CMV-3 mammalian expression vector (Sigma-Aldrich, St Louis, Missouri, USA). In addition, pVL530P- was used as a source of the human α 3(IV)NC1 P- sequence (the P- sequence within the pVL530 vector, map not shown as restriction digestion not used and the vector not used).

2.2. – Production, purification and use of protein L

2.2.1. – PCR for protein L fragment

The protein L PCR reaction was set such that 1µl (430ng) source plasmid (Ulf Sjöbring, University of Lund) was diluted 1:50 to give 8.6ng/µl, and 21.5ng of this DNA was used to amplify the required fragment by PCR with the addition requisite restriction sites. The primers were used at a stock concentration of 10µM. The primers were designed to also include various restriction sites and were named PL1 (forward primer) containing Eco RI (bold, underlined) and Nco I (Bold, italic) restriction sites and PL2 (reverse primer) containing Bam HI (italic) and Kpn I (underlined). The primer sequences were subjected to BLAST search to ensure no cross-reactivity.

PL1: 5'–**GGAATTCCATGGAAAATAAAGAAGAAACACC**– 3'

PL2: 5'–GGAATTCGGATCCCCGGTACCGTTTTTGTCAACTTTCTTACCTG–3'

The PCR programme was set up such that there included a hot start and a final, prolonged elongation step.

Specific conditions:

94°C for 10 min

94°C for 60 s	}	for 30 cycles
50°C for 60 s		
72°C for 90 s		

72°C for 5 min then 4°C for 5 min.

Following PCR, a 900bp fragment could be seen and purified from 1.5% agarose gel using PCR clean up kit (Qiagen).

After frequent transformation failure using Nco I and Bam HI digested pET25b with similarly digested protein L, the fragment was sequenced to check whether there was a problem with the PCR fragment being amplified using the Protein L primers. To do this, the purified protein L fragment was ligated into pGEM-T (a commercial vector designed to accommodate all PCR fragments with a single “A” overhang). This ligation was carried out according to the pGEM-T datasheet, and transformed into *E. coli* as described in section 2.4. Colonies were then grown up for miniprep, then midiprep (Qiagen midiprep kit) before being sent for sequencing analysis. The sequence of the protein L fragment was correct, however the reverse primer, PL2, was lacking the Bam HI restriction site and contained some extra bases, explaining why all subsequent digestions failed.

To try to recover the fragment, rescue oligos were designed, containing *Kpn I* and Sac I restriction sites.

PL2rescuetop: 5’-ATGGTACCAGCGCTAGCTCGAGCT-3’

PL2 rescuebottom: 5’-CGAGCTAGCGCTGGTACCATGTAC-3’

These were resuspended to a final concentration of 100µM and annealed. Equal volumes were mixed, heated to 99°C for 10 min, and gradually cooled to 30°C over 30 min. These annealed oligos could then be digested with Kpn I and Sac I and ligated into similarly digested pGEM-T-protein L. Following this ligation, the pGEM-T ligations were transformed into DH5 *E. coli* as previously described (section 2.4), and test digested using Nco I and Nhe I. Colonies with the correct

sized fragment were grown up for midiprep, digested with Nco I and Nhe I, then ligated into pET25b.

2.2.2. – Production of protein L

E. coli BL21 cells were transformed as described in section 2.6.2., such that they contained the correct pET25b-protL plasmid. These were grown overnight on inverted LB-ampicillin plates, and colonies were picked to test for protein production (section 2.1.). The most productive colony was used as a starter culture for a larger (2l) preparation and to use as a glycerol stock (section 2.10.3.).

As for the bacterial recombinant $\alpha 3(\text{IV})\text{NC1}$, the 2L culture was grown in LB broth with 50 $\mu\text{g/ml}$ ampicillin, to an optical density of 0.6 (520nm), then induced to produce the protein L for 3 h by adding 1mM IPTG. After induction, the cell suspension was centrifuged at 2200g for 30 min, 4°C (Sigma 4K15), and the pellet frozen to crudely lyse the cells.

2.2.3. – Production of a Glycerol Stock

The glycerol stock was made by concentrating 4ml uninduced bacterial culture into 2ml LB with 50 $\mu\text{g/ml}$ ampicillin, and adding 500 μl 80% glycerol in LB. This, once mixed well, was snap frozen in aliquots in liquid nitrogen and stored at -80°C.

2.2.4. – Immunoglobulin G-linkage to cyanogen-bromide activated sepharose beads.

Cyanogen bromide-activated sepharose beads were linked to human IgG as recommended by the manufacturer. In brief, 5g beads were swelled in sequential washes of 250ml ice-cold 1mM hydrochloric acid. These were then washed with

175ml deionised water, filtered, and resuspended in 25ml 0.1M sodium hydrogen carbonate, 0.5M sodium chloride, pH 8.4. To this solution, 1g purified human immunoglobulin was added and incubated on a rotor mixer at 4°C for 48 h. The beads were spun at 700g for 5 minutes (Sigma 4K15) and the supernatant removed. The beads were then washed in 0.1M sodium hydrogen carbonate, 0.5M sodium chloride, pH 8.4 and spun 5 times. After the final spin, the pellet of beads was resuspended in 0.2M glycine, pH 8 and left to block on a rotor mixer at 4°C for 18h. The beads were packed into a glass column using a peristaltic pump and washed with alternating buffers containing 0.1M sodium hydrogen carbonate, 0.5M sodium chloride, pH 8.4, or 0.1M sodium acetate, 0.5M sodium chloride, pH 4. The column could then be used for protein L purification.

2.2.5. – Purification of protein L

Prior to use, the column was rinsed with alternating buffers containing 0.1M sodium hydrogen carbonate, 0.5M sodium chloride, pH 8.4, and 0.1M sodium acetate, 0.5M sodium chloride, pH 4, then equilibrated with 0.1M sodium hydrogen carbonate, 0.5M sodium chloride, pH 8.4. The protein L pellet was resuspended in PBS prior to application to the column (10ml), and fractions of 25ml were collected of post-load, during washing (with PBS, pH 7.4), then elution with 100ml 0.1M glycine, pH 2. The column was then further washed with PBS and 0.1M sodium hydrogen carbonate, 0.5M sodium chloride, pH 8.4. During each step, 25ml fractions were collected and an aliquot from each was run on both an acrylamide gel and coomassie-stained, and transferred for western blotting and probing with anti-HSVtag antibody (1µg/µl, 1:100 dilution). The elution fractions were neutralised to

pH neutral pending analysis by western blot to determine the location of the protein L.

2.2.6. – Labelling protein L with FITC

In order to FITC label, the protein L, the IgG-linked column was used to immobilise the protein L while 2ml 1.5mg/ml FITC in DMSO was slowly run through the column. The column was then incubated in the dark for 1 h and washed with 1L PBS, the protein L was eluted with 0.1M glycine, pH 2 and the column further washed with PBS, refreshed with 0.1M sodium hydrogen carbonate, 0.5M sodium chloride, pH 8.4, and 0.1M sodium acetate, 0.5M sodium chloride, pH 4, and filled with 1M sodium chloride for storage at 4°C. The protein L-FITC was neutralised and ethanol precipitated before resuspension at 0.6mg/ml in PBS.

2.2.7. – Flow cytometry with FITC-labelled protein L on B cells

An EBV-transformed B cell line, positive for HLA DR15 was grown in normal R10 media. The cells were counted and 2×10^5 cells were stained for each reaction. The cells were double stained with anti-CD19-PE (1:50 dilution), washed with PBS, then stained with protein L-FITC (1.2µg, 3µg or 6µg), washed well in PBS and read on a Becton Dickinson FACScan (BD Biosciences, San Jose, California, USA). Negative controls were the IgG₂-PE isotypic control (1:50 dilution) and unlabelled protein L, while each staining condition was measured in triplicate.

2.2.8. – Confocal microscopy using protein L-FITC to test binding on B cells

The DR15-positive B cell line used in flow cytometry experiments was also used for confocal microscopy and imaging of protein L-FITC binding to cells. The

cells were resuspended at 5×10^6 /ml, and staining was carried out in round-bottomed 96-well plates. In total, 3 different concentrations of protein L-FITC (1.2 μ g, 3 μ g or 6 μ g) were used to label the cells for three different time points (15, 30 and 60 min), with one well remaining unstained. At all points, the plate was protected from light as much as possible. Each well was then washed well with PBS, and the cells were fixed with 4% paraformaldehyde for 30 min at room temperature. After a further wash with PBS, the cells were counterstained with a nuclear stain, DAPI (1 μ g/ml in methanol, diluted 1:100 in PBS) for 2 min before washing well with PBS. The cells were then mounted onto glass slides and subjected to confocal microscopy (Zeiss LSM510meta).

2.3. – Genotyping polymerase chain reactions (PCRs)

As the laboratory had had problems with the Taq enzyme source for our department, all the PCR reactions described in this thesis, unless stated otherwise, used illustra PuReTaq Ready-To-Go PCR beads – tubes containing a single bead. Each bead contained lyophilised 2.5 IU PuReTaq DNA polymerase, 10mM tris, 50mM potassium chloride, 1.5mM MgCl₂, 200 μ M dNTPs and BSA, such that the DNA, primers and water could be added directly to the tube and the reaction started (GE healthcare). The PCR machine used was the ATC 201 from Nyxtechnik.

2.3.1. – Extraction of DNA from mouse tissue

Earmarks were used both to identify individual mice and as a source of tissue for DNA extraction and subsequent genotyping. Earmarks were digested for a minimum of 8 h with 100µg proteinase K in 200µl lysis buffer (0.2% SDS, 0.1M Tris, 5mM EDTA, 200mM sodium chloride) at 55°C. This was then centrifuged at 18000g for 10min (Sigma 3-15K) and the supernatant carefully transferred to a new Eppendorf tube. This was then incubated with isopropanol for 30 minutes, centrifuged and the pellet washed with 70% ethanol. After further centrifugation, the supernatant was aspirated and the pellet allowed to air dry before resuspension in 200µl 10mM Tris, pH 8.5.

2.3.2. – PCR for mouse class II

These reactions test for the wild type mouse class II gene and the knocked out version of the gene (contains a neomycin cassette). In the wild type reaction IAB (WT), (ABOL2 and ABOR2 primers), two fragments (169bp and greater than 1000bp) were expected, while in the knock out (KO) reactions, Neo (Neo and ABOL2 primers) and NeoR (NeoR and ABOR2 primers), fragments of 340 and 800bp were expected respectively. Two µg DNA were used per reaction. Primers were used at a stock concentration of 10µM (final conc 0.4µM) and were designed as follows:

Neo: 5'- GAG GAT CTC GTC GTG ACC CA-3' (forward)

ABOL2: 5'- CAC CAA CGG GAC GCA GCG CAT AC-3' (reverse)

ABOR2: 5'- GGC CCT CGT TCG CTC CAG GAT CT-3' (forward)

NeoR: 5'- GCG ATA CCG TAA AGC ACG AG-3' (reverse)

Conditions for IAB (WT) PCR:

75°C - 10min, hot start

95°C - 1min, denaturing }
 67.8°C - 1min, annealing } 30 cycles
 72°C – 90 s, elongation }

72°C for 10min then 4°C for 10 min

Conditions for Neo and NeoR (KO) PCRs:

75°C - 10min, hot start

95°C - 1min, denaturing }
 64°C - 1min, annealing } 30 cycles
 72°C – 90 s, elongation }

72°C for 10min then 4°C for 10 min

2.3.3. – PCR for human MHC class II, HLA-DR15

These reactions test for the inserted MHC class II double transgene (DRA and DRB). In the DRA reaction, a fragment of 124bp was expected, while in the knock DRB reaction, a fragment of 190bp was expected. Two µg DNA were used per reaction. Primers were used at a stock concentration of 10µM (final concentration 0.4µM) and were designed as follows:

TgDRAL: 5'- CTC CAA GCC CTC TCC CAG AG-3' (reverse)

TgDRAR: 5'- ATG TGC CTT ACA GAG GCC CC-3' (forward)

TgDRBR: 5'- CTG CAC TGT GAA GCT CTC-3' (forward)

TgDRBL: 5'- TTC AAT GGG ACG GAG CGG GTG-3' (reverse)

Conditions for DRA and DRB PCRs:

75°C - 10min, hot start

95°C - 1min, denaturing
64°C - 1min, annealing
72°C – 90 s, elongation

} 30 cycles

72°C for 10min then 4°C for 10 min

2.3.4. – PCR for $\alpha 3$

These reactions test for the WT $\alpha 3$ (IV) gene and the KO version of the gene (contains a neomycin cassette), in the WT reaction, a fragment of 1000bp was expected, while in the KO reaction, a fragment of 850bp was expected. Two μ g DNA were used per reaction. Primers were used at a stock concentration of 10 μ M (final concentration 0.4 μ M), and 4A3 KOWTL is the reverse primer for both reactions. In addition, 1 μ l 25mM MgCl₂ was added to each reaction to give a final concentration of 2.5mM MgCl₂ for these reactions.

Primers:

4A3 KOWTL: 5'- ACG ACC TTT GTT AAA CTA GAA GAA GTC-3' (reverse)

4A3 WTR: 5'- TTC CCC TGT CAC CAG GAT TTC CC-3' (forward)

4A3 KOR: 5'- TGC TAA AGC GCA TGC TCC AGA CTG C-3' (forward)

Conditions:

75°C - 10min, hot start

95°C - 1min, denaturing
60°C - 1min, annealing
72°C – 90 s, elongation

} 30 cycles

72°C for 10min then 4°C for 10 min

2.3.5. – Visualisation of bands

The PCR reaction bands were visualised by running the reaction mixture on a 1.5% agarose gel containing 50µg ethidium bromide and made using 89mM Tris Borate, 2mM EDTA (177) solution. The gels were run at 100V for 30 min using a Mini Easigel Gel Electrophoresis System (VWR International) and visualised using a UV transilluminator (Uvidoc) and camera (Uvitec).

2.4. – Flow cytometry of MHC class II on mouse lymphocytes

Lymphocytes from the NTxDR15 transgenic mouse line were tested, by flow cytometry, for expression of the mouse class II molecule and the human HLA DR15 molecule, and compared with lymphocytes from a non-transgenic C57Bl/6J mouse. Lymph nodes were removed from each mouse and disaggregated by mashing through a 40µm cell strainer. The cells from each mouse were counted and resuspended at 2×10^6 /ml in HBSS. Both the anti-human DR15-FITC (0.1mg/ml, AbD Serotec) and the anti-mouse class II-FITC (0.1mg/ml, AbD Serotec) antibodies were used at 1:20 dilution and incubated on the cells for 30 min, room temperature, in the dark, prior to washing with 100µl HBSS and reading on the Becton Dickinson FACScalibur.

2.5. – Production of bacterial recombinant antigen

2.5.1. – Culture and induction of recombinant $\alpha 3(IV)NC1$ in bacteria

A glycerol stock containing transformed *E. coli* BL21 bacteria containing the pET25b- $\alpha 3(IV)NC1$ plasmid (produced by Dr R.G. Phelps, (165)) was used to streak an LB-agar-ampicillin (50 μ g/ml) plate and grown inverted overnight at 37°C. Colonies were picked and grown into 5ml LB –ampicillin starter cultures. 3ml was used to test protein production by induction with 1mM IPTG. The colonies were assessed for production of $\alpha 3(IV)NC1$, by protein concentration on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE), using a 12% acrylamide gel and the Laemmli buffer system (178), with the gel then stained with Coomassie blue. The colony with the greatest production of $\alpha 3(IV)NC1$ was used as the starter culture for a larger (2L LB-50 μ g/ml ampicillin) preparation. When at an optical density of 0.6, the bacteria were induced with 1mM IPTG for 3h, in an orbital shaker (200rpm, 37°C).

2.5.2. – Purification of bacterial recombinant antigen

The induced cultures were spun down at 2200g, 4°C, 30 min (Sigma 4K15) and the pellets resuspended in 35ml ice cold PBS, before sonication (Ultrasonic processor, Jencons) for 2 min on 50% duty cycle (sonicator at this setting only sonicates the sample in pulses equivalent to 50% of total time). This was then incubated with 0.2mg/ml HEL at 4°C for 1h on roller, then 2 μ g/ml DNase, 2mM MgCl₂ and 0.1% deoxycholic acid were added and incubated at 4°C, with constant movement, overnight. These suspensions were centrifuged at 9500g, 4°C for 30min (Centrikon T-42K) and the supernatant discarded before the pellets were resuspended in 30ml PBS, sonicated for 60s on 50% duty cycle and spun at 9500g, 4°C for 25min

(Centrikon T-42K). Again, the supernatant was discarded and the pellet resuspended in 25ml 0.5% triton X-100, 5mM Tris, and sonicated for 60s on 50% duty cycle and left on roller at 4°C for 30min. This was then spun at 9500g, 4°C, for 25min (Centrikon T-42K) prior to washing the pellet 3 times with PBS, sonicating between washes to resuspend the pellet. The pellet was weighed and resuspended in 6M guanidine, 50mM tris and incubated at 4°C on roller platform overnight.

To purify the recombinant $\alpha 3(\text{IV})\text{NC1}$, an IAA bead column was used. The column was rinsed with 50mM Tris, pH 7.5 and loaded with 0.1M nickel chloride. The column was then washed twice with 50mM Tris, pH 7.5, followed by three washes with 6M guanidine hydrochloride, 50mM Tris, 20mM Imidazole, pH 7.5. 5ml of the $\alpha 3(\text{IV})\text{NC1}$ preparation was applied to the column and allowed to drip through the column under gravity. The column was then washed a further three times with 6M guanidine hydrochloride, 50mM Tris, 20mM Imidazole, pH 7.5, before the protein was eluted with three applications of 2ml 6M guanidine hydrochloride, 50mM Tris, 100mM EDTA, pH 7.5. The column was washed with 20ml 50mM Tris, pH 7.5 and reloaded with nickel chloride for the remaining $\alpha 3(\text{IV})\text{NC1}$ guanidine solution to be purified. The eluted protein was ethanol precipitated and the pellet resuspended in 8M urea.

Once resuspended in the urea, the protein concentration was measured by SDS PAGE using a 12% acrylamide gel and the Laemmli buffer system (178). The gel was coomassie stained for 1 h before washing and checking the amount of contamination and the approximate concentration of the protein. The protein solution was also subjected to spectrometry (280nm) so a more precise concentration

could be determined. The remaining protein solution (recombinant human $\alpha 3(\text{IV})\text{NC1}$, $\text{rho}\alpha 3$) was then frozen in aliquots at -20°C .

2.6. – Extraction of $\alpha 3(\text{IV})\text{NC1}$ from tissue

2.6.1. – Disaggregation of tissue and disruption of cells

Tissue was disaggregated as previously described by Spiro (179) and Borza *et al.* (168). In brief, cortex was sliced off semi-frozen human kidneys (after careful decapsulation) and sliced into small strips. Sieves (Endecotts) were stacked such that the order of mesh size is (top to bottom) 150 μm , 63 μm , 250 μm . Cortex strips were added a little at a time into top sieve and mashed through with base of a conical flask and washed thoroughly with PBS. Glomeruli collect on 63 μm sieve, while tubules go through it. All sieves were washed well to ensure maximal yield. Glomeruli were washed twice with PBS and spun at 700g at 4°C for 30min (Sigma 4K15). Supernatants were discarded and the pellet resuspended in 10ml PBS then frozen at -20°C .

The glomerular pellet was thawed then sonicated (Ultrasonic processor, Jencons) for a total of 2.5min (5 times 30s) (50%), output dial on 4, per tube. The matter was checked microscopically for glomerular disruption and lack of cells, then was washed 3 times with PBS, with each centrifugation step at 700g, 15min, 4°C . The pellets were then washed 3 times with deionised water, each spin at 700g,

15min, 4°C (Sigma 4K15). After the final spin, the supernatant was discarded and the pellet frozen.

To extract the NC1 domains, the glomerular preparation was collagenase-digested. To the thawed glomerular preparation, 0.7% type VII collagenase and 10% v/v 10x collagenase buffer (0.1M Tris, 0.05M calcium acetate, pH7.4) were added and the resultant solution incubated at 37°C on orbital shaker for 5h. After the digestion, the NC1 domains are solubilised so the solution was spun at 700g, for 30min, at 4°C and the supernatant was carefully removed and kept frozen prior to purification by cation exchange chromatography.

2.6.2. – Purification of human $\alpha 3(IV)$ NC1 by cation-exchange chromatography

For cation exchange chromatography, sepharose beads were used in a 1ml column on a Biocad HPLC machine. The programme for isolation of the human $\alpha 3(IV)$ NC1 domains was optimised using HEL and BSA – two proteins with different atomic masses and ionic characteristics. The column was equilibrated with 10ml buffer A (2M Urea, 0.05M sodium acetate, pH5) prior to sample loading. Subsequently, the column was washed with 2ml buffer A before implementation of an automatic pH gradient of 100% buffer A to 100% buffer C (2M Urea, 0.05M Tris, pH10) during 15 column volumes (final volume approximately 15ml). Buffer C was used to wash the column for a further 4ml, before a salt gradient was implemented over 15ml (100% buffer C to 100% buffer B (2M urea, 0.05M Tris, 1M sodium chloride, pH10)). 4ml Buffer B was used to wash the column, before cleaning the column with buffer A for 15ml. The flow rate was 5ml/ minute, the sample size was 5ml, and 5ml fraction collection was begun immediately after sample injection.

Each fraction was ethanol precipitated and the fractions containing peaks were tested for the presence of human $\alpha 3(\text{IV})\text{NC1}$ (hu $\alpha 3$) by western blotting.

The western blotting was carried out using the Laemmli buffer system on a 12% acrylamide gel (178), before transfer to nitrocellulose membrane. The membrane was blocked using 5% milk in PBS with 0.1% Tween, probed with Goodpasture patient sera (1:40 ratio of sera to milk solution), washed (PBS with 0.1% tween) then probed with a secondary, alk phos-conjugated anti-human IgG polyclonal antibody (1:10 000 ratio in milk solution). The transfer was washed as above and visualised using BCIP and NBT.

2.7. – Production of $\alpha 3(\text{IV})\text{NC1}$ antigen by eukaryotic cells

2.7.1. – Production of construct for antigen production

The pVL530P- was used as a source for the human $\alpha 3(\text{IV})\text{NC1}$ sequence and amplified by polymerase chain reaction (PCR) using the following primers:

A3BglnlyF: 5'- CCAG**ATC**CTGAAAGGAAAACGTGG

HA3EXP2R2: 5'- CCATG**GTACCTCGAG**TTCTGCTGTC

A3BglnlyF contained a Bgl II (bold) restriction site, while HA3EXP2R2 contained the Nco I (italic), Kpn I (italic, underlined) and Xho I (bold, italic, underlined) restriction sites. The PCR reaction was carried out using PCR beads, 10 μ M stock primer and 84.8ng DNA. The PCR conditions were optimised such that: 75°C – hot start

94°C – 1 min, denaturing	} 30 cycles
65°C – 1 min, annealing	
72°C – 30 s, elongation	

72°C – 5 min, then 4°C – 5 min.

This programme successfully amplified a fragment of 900bp, which was then extracted from an agarose gel using the GE healthcare illumina kit as per the manufacturers instructions (GE Healthcare, Buckinghamshire, UK). The PCR product was cloned into the TOPO blunt end vector as per vector instructions, and transformed into commercially sourced NovaBlue singles (Novagen, Merckbiosciences, Darmstadt, UK).

Colonies were grown in a 5ml LB culture for miniprep, the DNA extracted and test digested with Bgl II and Nco I. The expression vector pFLAG-CMV-3 was digested simultaneously and the two digests were ligated prior to transformation into NovaBlue singles.

Again, colonies were picked for miniprep, test digested with Bgl II and Nco I, and positive digests were grown further for midiprep using a commercially available kit (Qiagen, Crawley, UK) as per manufacturers recommendations.

2.7.2. – Transformation of *E. coli* DH5 cells

Transformation of *E. coli* DH5s to produce pFLAG- α 3(IV)NC1 P- was carried out using standard techniques for making and transforming competent cells. In brief, DH5s were grown in a 50ml LB culture to an optical density of 0.3-0.4 (520nm). The cells were centrifuged at 2900g, 4°C for 10 minutes (Sigma 4K15), the supernatant decanted and resuspended in 15ml ice-cold transformation buffer (100mM potassium chloride, 45mM tetrahydrous manganese chloride, 10mM

dihydrous calcium chloride, 3mM hexamine cobalt chloride, 10mM morpholinoethanesulphonic acid). After incubating the cell suspension on ice for 15 minutes, the centrifugation step was repeated and the cells then resuspended in 2.5ml ice-cold transformation buffer. Immediately after resuspension, 140µl DTT- DMSO solution (1M DTT, 90% DMSO, 10mM potassium acetate) was added to the cells, mixed by swirling and chilled on ice for 10min. A further 140µl DTT-DMSO solution was added and swirled, then chilled on ice for 20 minutes. Into 1.5ml Eppendorf tubes, 5µl plasmid was pipetted, plus 50µl cell suspension and chilled on ice for 30 minutes prior to heat shock (42°C water bath, 90 seconds) and placed back on ice for a further 2 minutes. To each tube, 200µl SOC media (2% casein, 0.5% yeast extract, 10mM sodium chloride, 2.5mM potassium chloride, 20mM MgCl₂/magnesium sulphate, 10% glycerol) was added and incubated at 37°C for 45 minutes. This was then spread onto LB agar plates containing 50µg/ml ampicillin and incubated for 18 hours.

Colonies were then picked from the plate, and grown for a 5ml miniprep culture in order to amplify the amount and extract plasmid DNA. Of this culture, 3ml were used to extract DNA, with the remaining 2ml kept in case of need for repetition or for a starter culture. For the miniprep, 3ml of culture was centrifuged at 10000g for 3 min (Sigma 3-15K). The supernatant was discarded and the pellet resuspended by vortexing in 100µl Glucose – Tris – EDTA buffer (50mM glucose, 25mM tris, 10mM EDTA). With the pellet resuspended, 200µl 0.2M sodium hydroxide, 1% SDS was added, mixed by inversion and chilled on ice for 5 minutes. When chilled, 150µl 3M potassium, 5M acetate solution was added, mixed by vortexing and chilled for a further 15 minutes on ice. The tubes were then

centrifuged at 10000g for 5 min (Sigma 3-15K) and the supernatant carefully decanted to a new Eppendorf tube. To precipitate protein out of the solution, 200µl phenol was added, mixed by vortexing and centrifuged at 10000g for 2 minutes (Sigma 3-15K). The upper phase was removed and pipetted carefully into a new Eppendorf tube with an equal volume of isopropanol, mixed by vortexing and centrifuged at 10000g for 5 min (Sigma 3-15K). The pellet was kept and washed with 1 ml 70% ethanol, centrifuged at 10000g for 5 minutes (Sigma 3-15K), and the supernatant carefully decanted. The remaining pellet was allowed to air dry prior to resuspension in 10mM Tris, pH8.

2.7.3. – Transfection of human embryonic kidney 293 cells

HEK293 cells were grown to confluence, then seeded at 1.5×10^5 into 6 well plates and allowed to grow. When the wells were 80% confluent, they were washed well with HBSS and 1ml minimal media (DMEM) was then added.

DNA was diluted to 0.75µg/µl and 2µl was pipetted into 98µl minimal media. Lipofectamine was diluted 1:25 in minimal media, allowing 100µl per well for transfection. The DNA and Lipofectamine solutions were mixed and incubated at room temperature for 30 min. A further 200µl minimal media was added to each transfection mixture (400µl final volume).

The media was removed from the cells and replaced with 400µl minimal media and 400µl lipofectamine-DNA mixture, then incubated for 5h at 37°C. Following the incubation, the lipofectamine was removed and replaced with 1ml DMEM media (DMEM, 10% HI-FCS, 1mg/ml L-glutamine, 1.2mg/ml penicillin/streptomycin). The following day, the transfected cells were trypsinised and resuspended in

selective media (DMEM with 0.4mg/ml geneticin) at serial dilutions. As the tranfects grew, they were tested for $\alpha 3(\text{IV})\text{NC1}$ expression by incubating with minimal media for 12h. This media was then subjected to western blot and probed with patient sera to test for $\alpha 3(\text{IV})\text{NC1}$ protein production.

2.8. – Lymphocyte stimulation assay

Mice of the relevant genotypes were given subcutaneous immunisations with 50 μg antigen (rh $\alpha 3$, hu $\alpha 3$ or ovalbumin) emulsified with complete Freund's adjuvant by sonication (0.5 $\mu\text{g}/\mu\text{l}$ antigen in PBS, plus equal volume adjuvant, sonicated 3 times, for 30s on 50% duty cycle), left for 7 days, then given a booster immunisation containing the antigen emulsified (by sonication, as above) with incomplete Freund's adjuvant. After a further 7 days, the mice were culled and the lymph nodes extracted for mechanical disaggregation.

Cells from the disaggregated lymph nodes were resuspended in 3ml DMEM containing 10% HI-FCS, Penicillin/Streptomycin and L-Glutamine. The cells were counted and diluted to $1 \times 10^6/\text{ml}$ and 1×10^5 plated/well 96 well round bottomed plate. Cells were stimulated with 100 μl non-specific protein Ovalbumin (20 $\mu\text{g}/\text{ml}$), T cell mitogen Con A (5 $\mu\text{g}/\text{ml}$), peptide AS345 (20 $\mu\text{g}/\text{ml}$), peptide AS346 (20 $\mu\text{g}/\text{ml}$), rh $\alpha 3$ (50, 100 or 200 $\mu\text{g}/\text{ml}$). In addition to the aforementioned proteins and peptides, the cells were stimulated with overlapping 15mers of the $\alpha 3(\text{IV})\text{NC1}$ domain, $\alpha 3(\text{IV})\text{NC1}$ -9-5 – $\alpha 3(\text{IV})\text{NC1}$ 221-235 (50 $\mu\text{g}/\text{ml}$), the sequences with start and finish amino acid positions of each peptide are described in detail in Appendix I.

Each stimulant or peptide was plated in triplicate with the final concentrations of stimulant listed above and a final cell count of 1×10^5 per well. The plates were incubated at 37°C, 5% CO₂, for 72h, prior to pulsing for 18h with 0.5µCi ³H-thymidine. The cells were harvested onto filters using a Tomtec cell harvester and the tritium incorporation counted using a β-Counter (Wallac 1205 Betaplate reader).

2.9. – Haematoxylin and eosin (H&E) staining of tissue sections

Slides containing paraffin embedded kidney sections (made from formaldehyde-fixed kidneys isolated at same time as lymph nodes, and sectioned and mounted by Bob Morris in the histopathology laboratory within the University of Edinburgh) were placed in a slide holder (glass or metal) and deparaffinised in histoclear, then rehydrated using sequential dilutions of ethanol: 100%, 90%, 75% then water. Haematoxylin was skimmed to remove oxidised particles prior to staining. After the haematoxylin step, the slides were rinsed in deionised water and dipped fast in acid ethanol (70% ethanol, 0.25% glacial acetic acid) to destain, before further washing with water then deionised water. To counterstain the sections, the slides were dipped briefly in eosin prior to dehydrating the sections in increasing concentrations of ethanol (75%, 90% then 100%), before soaking in histoclear. Coverslips were mounted with DPX mounting medium, before being left to dry overnight in the fume hood. Photographs were taken using a Zeiss Axioskop 2 microscope at x400 magnification.

2.10. – Production of hybridomas

Mice with the genotype mouse class II knock out, DR15 positive, $\alpha 3(\text{IV})$ knock out were immunised with rh $\alpha 3$ using the same method as described in the lymphocyte stimulation assay (section 2.8.). Upon sacrifice, the lymph nodes were extracted and disaggregated. The cells were resuspended at 1×10^6 with 50 μg rh $\alpha 3$ in DMEM. After 3 days, cells were washed and plated at 4×10^5 with 20IU/ well recombinant IL-2 in DMEM.

In order to create a hybridoma, the T cell must be fused with an immortal cell line, in this instance BW5146 cells, a myeloma line. The different cell types were washed individually with HBSS, then counted and combined in equal numbers at 2.5×10^6 of each cell type in 15ml. After centrifugation (450g, 4°C, 5min, Sigma 4K15), the supernatant was aspirated and 700 μl PEG added dropwise over 90 sec (timed), with gentle tapping to mix. This was followed by 5ml HBSS added dropwise over 2 min (timed) and 15ml HBSS added dropwise over 3 min (timed), each stage mixing by gentle tapping. The cell suspension was then spun gently and the supernatant removed. To the pellet, 30ml R10 media (RPMI, 10% HI-FCS, 1.2mg/ml penicillin/strepomycin, 1mg/ml L-glutamine, 1 μl /ml media βMe) was added over 3 min (timed) with gentle tapping. 20ml of the resultant suspension was poured into a petri dish and 100 μl pipetted per well of 2, 96 well flat-bottomed plates (stock). To the remaining 10ml cell suspension, 20ml R10 media was added, gently mixed and 20ml was further pipetted at 100 μl /well into two plates (1:3 dilution). For the final dilution (1:9), a further 20ml R10 media was added, gently mixed and pipetted as for the previous plates. These plates (6 per fusion) were then incubated at

37°C for 24h, before 100µl HAT media (R10 plus HAT supplement) (180) was added to each well and incubated at 37°C.

Clones were picked after 7 days and expanded gradually to 8 wells of a 24-well plate, then into T25 flasks before weaning off HAT to HT (R10 plus HT supplement) media then to R10 media. The HAT then HT media provide selective components to encourage growth of the hybridomas and selectively kill unfused cells. The aminopterin is toxic unless the cells can bypass certain cellular mechanisms, so unfused myeloma cells and myeloma-myeloma hybridomas are selectively destroyed. The gradual weaning is required as the cells need to utilise different pathways for DNA synthesis in the presence and absence of aminopterin. The cells that are unable to utilise the salvage pathway do not survive, but those that do need time to readjust to using the main DNA synthesis pathway, as the two pathways are not both constitutively active.

Hybridomas were tested using the lymphocyte stimulation assay technique described in section 2.7., but instead of being tested against individual peptides, were tested against peptide sets with a final concentration of 50µg/ml overall. Each set contained 5 of the 47 overlapping 15mer peptides spanning the $\alpha 3(IV)NC1$ domain and are described in more detail in Appendix I. This was done to reduce the amount of individual peptide used, and to reduce the number of peptides for testing in a complete assay.

2.11. – Flow cytometry for Ig κ and Ig λ

The same EBV-transformed B cell line used for testing Protein L-FITC binding was also tested for Ig κ and Ig λ proportions. For this, 2×10^5 cells were stained, in triplicate, with either mouse anti-human Ig κ -FITC (4mg/ml, diluted 1:100), or mouse anti-human Ig λ (1mg/ml, diluted 1:10) and goat anti-mouse IgG-FITC (1mg/ml, diluted 1:100). Each antibody was incubated on the cells for 30 min at room temperature and the cells were washed between antibodies in HBSS, before running on the Becton Dickinson FACScalibur (BD Biosciences, San Jose, California, USA).

Chapter 3 – Use of Protein L to improve antigen uptake into B cells

3.1. – Introduction

In order to activate T cells, B cells bind whole antigen through the B cell receptor (BCR) and internalise the molecule for processing into fragments that can bind MHC class II molecules. Uptake of antigen and presentation of the processed peptides are linked. If the uptake process could be manipulated such that an excess of a specific antigen enters the APCs in use, it could be argued that any immune responses by specific hybridomas are directly linked to processing and presentation, as they will respond to a specific peptide fragment in proportion to the amount of it that is presented on the APC surface (181). One method to augment cellular uptake is to develop fusion proteins that can be linked to the protein of interest (181-183). These fusion proteins frequently include elements of the immune system, for example, ligands for B cell surface molecules, and have included different C3 protein fragments from the complement cascade (184). To improve uptake of the $\alpha 3$ molecule into HLA-DR15 specific B cells, it was hypothesised that $\alpha 3$ or $\alpha 3$ fragments could be linked to a B cell surface ligand to make a fusion protein able to access receptor-mediated uptake pathways.

Fusion proteins can be defined as novel proteins made by molecular engineering and have many possible applications beyond those described below.

When debating the use of a fusion protein, there are several things to consider, including the purpose of the protein, the type of target cell and how the cells would react to the fusion protein, and how to determine efficiency of binding and measure cellular responses. Here, the fusion protein is used to improve cellular uptake of antigen, and to enable targeted labelling under physiological conditions. Proteins from the immune system lend themselves particularly well to these scenarios as, broadly speaking, the immune system recognises antigen, enabling endocytosis and processing of the antigen, and development of further immune responses where required. Two classical examples of proteins used as fusion proteins include the Fab fragment of immunoglobulin, and the complement protein C3. Examples of their usage include using the Fab fragment targeted against CD19 linked with superantigen to induce uptake and lysis of B cells (185), or targeted against CD86 to induce uptake into neutrophils (186). C3 fragments have been used to target T cells (187), or linked to colloidal gold to track uptake of antigen into B cells by electron microscopy (184). Carbohydrate moieties have also been suggested to improve uptake into cells – Adams *et al.* produced a high mannose carbohydrate bound to ovalbumin to determine uptake by dendritic cells (188) and that resulted in the antigen entering either the MHC class II processing pathway or the MHC class I processing pathway, and the carrier therefore having increased versatility for use in generating broad-spectrum T cell responses.

While these proteins (and carbohydrates) remain a very useful tool for focussing and improving purification, uptake or measurement of uptake, for this project, it was decided that characteristics seen in a relatively newly discovered bacterial protein, protein L, could be exploited (189). Protein L is a marker of

virulence expressed by 10% of the most infectious strains of *Peptostreptococcus magnus*. It was first identified in 1988 and is a possible candidate for use as a fusion protein to augment uptake of antigen into B cells because of its binding specificity. It binds with high affinity to the extracellular part of membrane bound immunoglobulin, in particular the Ig κ chains (specifically, the variable light chain (190)), but not to the antibody-binding fragment. Protein L also binds Ig λ variable light chains, but with lower affinity. Since the majority of human B cells (approximately 60%) express Ig κ (the remaining ~40% being Ig λ), this mechanism could be exploited to improve the uptake of α 3 peptides into the DR15 positive B cells, by bringing the antigen fragment of choice in close proximity to the BCR (figure 3.1.1).

The actual structure of protein L is very simple and consists of a 4-stranded β -sheet packed with a single α -helix (191), with the binding domains on either side of each strand of the β -sheet. The protein consists of various domains, with Ig κ binding occurring through five highly homologous domains named B1-B5 as shown in figure 3.2.1. Each of these binding domains contains two binding sites for Ig κ , corresponding to the two edges of the β -sheet (192).

Protein L is also somewhat unusual since it demonstrates a large number of main chain interactions relative to side chain interactions (amino acid mutations at each point showed little effect on overall binding capacity) (192), and it has also been shown that binding through the B1-B5 domains is ambidextrous (meaning the Ig κ chain can bind on either side of the protein binding domains, irrespective of direction of the target binding site).

Figure 3.1.1. – Schematic to illustrate BCR-mediated uptake of antigen and how the protein L-antigen may interact with B cells

This schematic illustrates BCR mediated uptake of antigen into B cells (figure A) – whole antigens bind the BCR on the cell surface, the BCR: antigen complex is internalised and fuses with lysosomes to be processed and presented on the B cell surface in conjunction with MHC class II molecules. Adapted from Wang *et al.*(9). Figure B illustrates how the protein L-antigen complex may bind to the BCR, thereby augmenting BCR-mediated uptake.

Figure A

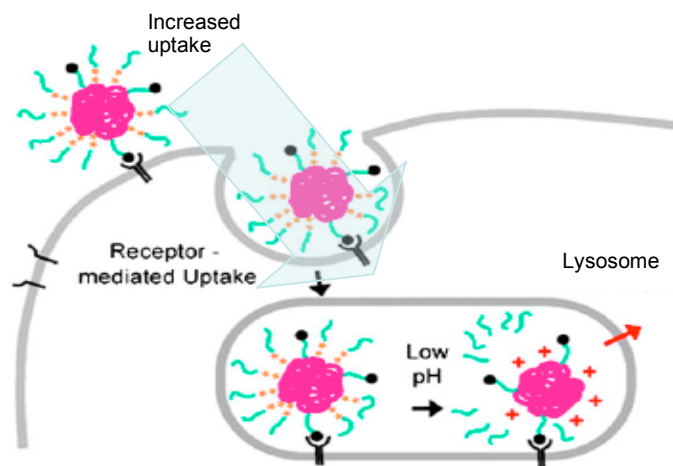
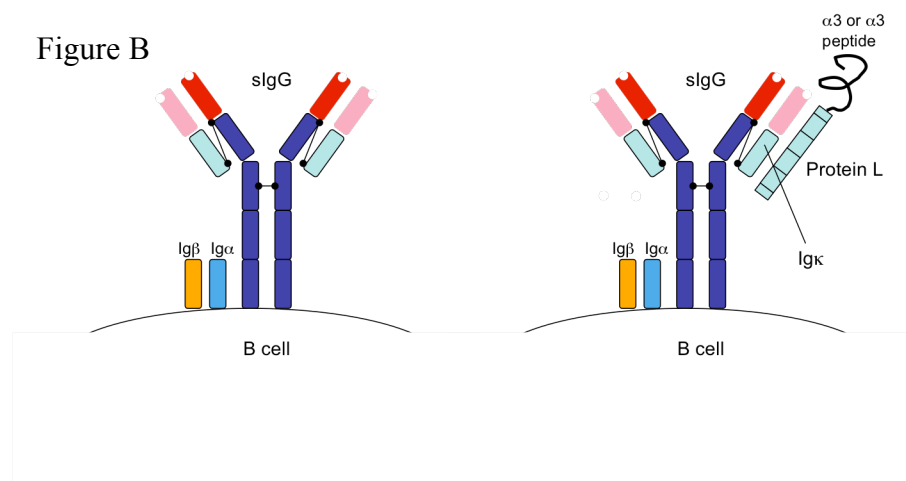


Figure B



This insensitivity to side chain interactions possibly explains how this protein can bind with high affinity to Igk light chains in different species and may have developed as an evolutionary adaptation in response to the development of immune systems of these different species.

These factors, plus the knowledge that the protein has already been sequenced and the binding domains B1-B4 cloned into a bacterial plasmid for recombinant protein production were an added incentive for determining the possibility of its usage as a fusion molecule. In addition, purification of protein L can be done through binding the protein to an IgG-sepharose column, so the protein can be washed and eluted into a known volume of buffer (as described in full in Chapter 2) with relative ease. This can reduce the specificity of the responses as a mass influx of immune cells flood the affected area in an attempt to eliminate the source of infection (184, 187). This chapter concentrates on the work undertaken to ensure the protein L was able to specifically bind and be taken up by B cells before ligating $\alpha 3$ proteins into the construct.

3.2.- Results

3.2.1. – Production of the Protein L construct

The cloning strategy for production of the protein L fragment illustrated in figure 3.2.1. and described in figure 3.2.2. involved digestion of the protein L construct obtained from Dr Ulf Sjöbring at the University of Lund (192), ligating the

protein L into pET25b, and subsequent manipulation to add either whole $\alpha 3(\text{IV})\text{NC1}$, or PCR products containing various fragments of the $\alpha 3(\text{IV})\text{NC1}$ protein, in particular $\alpha 3(\text{IV})\text{NC1}$ 136-150 and $\alpha 3(\text{IV})\text{NC1}$ 64-90, the fragments known to elicit immune responses in Goodpasture patients.

The initial protein L construct consisted of the protein L B1-B4 fragment within a vector (shown in figure 3.2.2). In order to insert the protein L into the pET25b vector and between the HSV and histidine tags, primers were designed to amplify the protein L fragment while incorporating the relevant restriction sites. The forward primer (PL1) contained the Eco RI (bold, underlined, italic) and Nco I (bold, italic) restriction sites, while the reverse primer (PL2) contained the Bam HI (italic) and Kpn I (underlined) restriction sites:

PL1: 5'- G**GAATTCATGG**AAAATAAAGAAGAAACACC

PL2: 5'- GGAATT**CGGATCCCCG**TACCGTTTTTGTCAACTTTCTTACCTG

These would produce the Protein L fragment with Eco RI and Nco I restriction sites at the 5' end, and Bam HI and Kpn I restriction sites at the 3' end. With appropriate digestion and ligation, this PCR product could be inserted into the pET25b vector.

Figure 3.2.1. – Schematic of the Protein L molecule.

Schematic of protein L structure illustrating different domains and functional regions. In this diagram, A represents the amino terminus, B1-5 represent the binding domain repeats, C signifies a region of as yet unknown function while W and M are the wall-spanning and transmembrane domains respectively. The region marked S contains a spacer region, while the region marked SS contains a signal sequence. Between the red lines is the region that was amplified by PCR and ligated into pET25b.

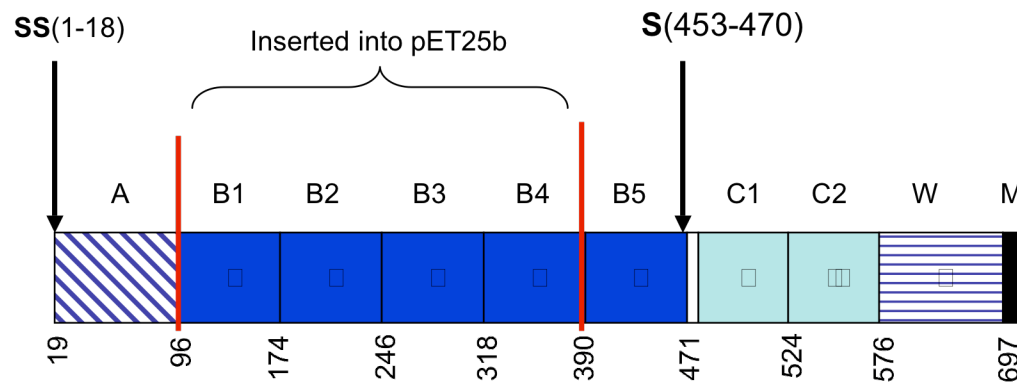
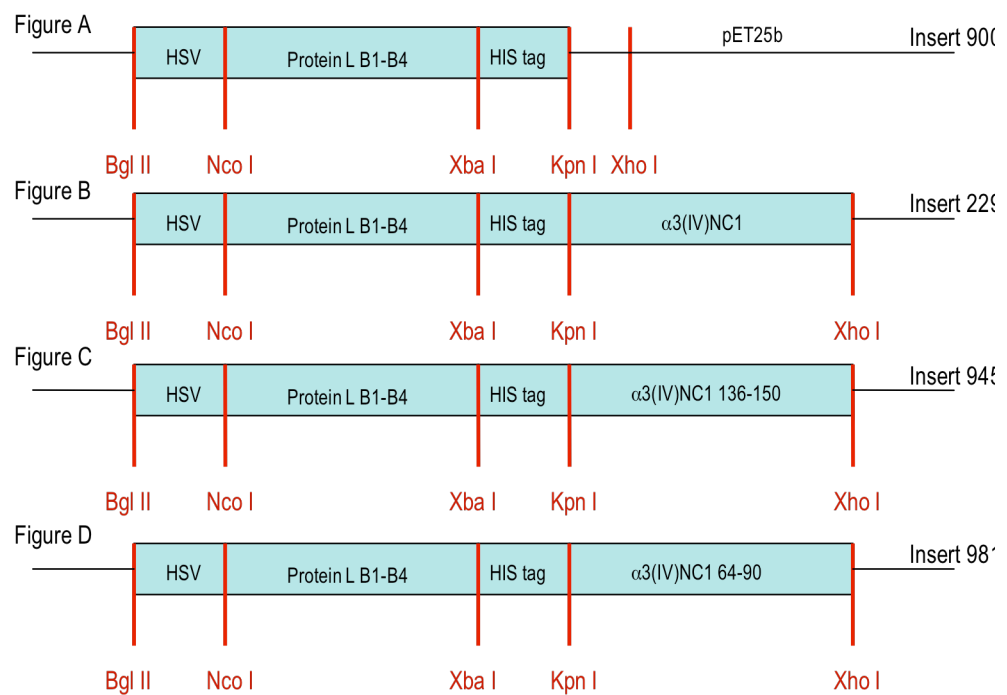


Figure 3.2.2. – Schematic of cloning strategy and potential fusion partners for Protein L

This schematic shows the inserted protein L B1-B4 sequence within the pET25b vector, flanked by the HSV leader sequence, a histidine tag, with added $\alpha 3(\text{IV})\text{NC1}$ (B), $\alpha 3(\text{IV})\text{NC1}$ 136-150 (C) or $\alpha 3(\text{IV})\text{NC1}$ 64-90 (D) as fusion partners. The red vertical lines mark restriction sites (for a restriction map of the pET25b plasmid see appendix II, for schematic of protein L in situ, see figure 3.2.1).



As described in sections 3.1. and 3.3., protein L can be utilised to improve uptake into B cells and investigate various aspects of antigen presentation by B cells. However, early cloning attempts failed due to a production error in the commercially sourced oligonucleotide primer, PL2. This highly unusual and unfortunately late-appreciated source of errors was finally determined by ‘blunt’ cloning the PCR product into pGEM-T (for restriction map see appendix II) and sequencing across the fragment.

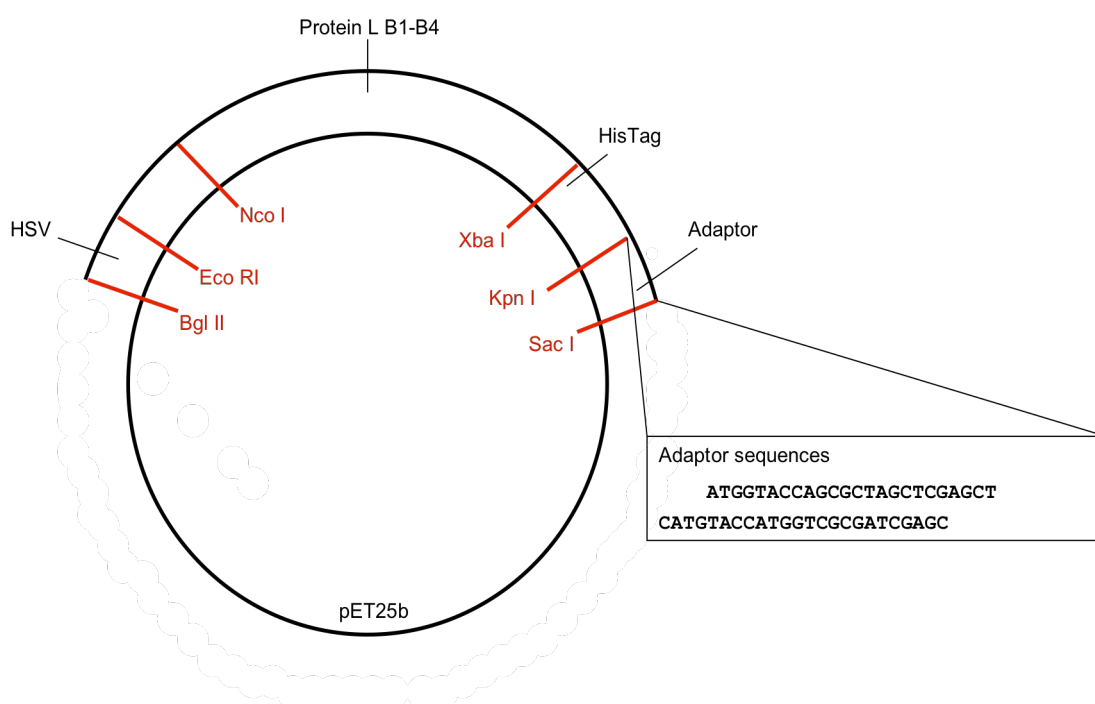
Adaptor oligonucleotide sequences were designed in order to insert additional restriction sites and correct the faulty construct, before protein production could begin (results figure 3.2.3. shows a schematic of the final pET25b vector containing the protein L insert), and the alterations made to the sequence during the scheme of work). The adaptor sequences were dephosphorylated and annealed such that:

5' - ATGGTACCAGCGCTAGCTCG***AGCT***
 3' - CATGTACCATGGTCGCGATCGAGC

These adaptors contained the Kpn I (underline, italics) and Sac I (bold, italics) restriction site overhangs, enabling the use of the faulty construct already generated by altering the restriction site to include Kpn I and Sac I.

Figure 3.2.3. – Schematic of the protein L construct as initially designed, with additional changes from adaptors designed to correct errors.

This diagram shows a restriction map of the pET25b plasmid with the protein L insert, plus the adaptors designed for insertion between the KpnI and Sac I restriction sites, in order to correct for the incorrect sequence derived from oligo synthesis. This schematic shows the areas that could be used for identification following purification (HSV and HisTag) plus the location of the adaptor sequence.



The corrected sequence was then digested using Nco I and Sac I, and ligated into pre-digested pET25b to create the construct shown in figure 3.2.3. Any subsequent manipulations to add $\alpha 3(\text{IV})\text{NC1}$ or $\alpha 3(\text{IV})\text{NC1}$ peptides used the pGEM-T construct as it proved easier to grow and use in bacterial transformations.

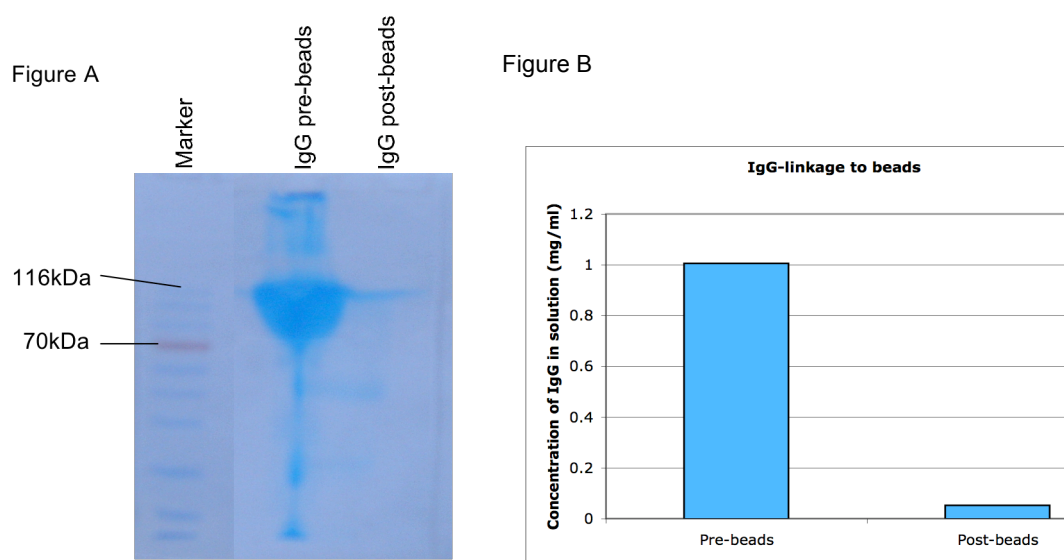
The protein L sequence with additional restriction sites was ligated into the pET25b expression vector (restriction map in appendix I) and transformed into *E. coli* BL21s – a bacterial strain that can be induced to produce protein in response to the addition of IPTG in the culture medium. This protein was not secreted into the surrounding media, so a considerable amount of purification was required before the protein preparation was suitable for use in cellular systems (for FACS or confocal microscopy).

3.2.2. – Purification of the protein L fusion protein

In order to purify the protein produced in this bacterial system, it was necessary to lyse the cells producing it, so I decided to utilise the ability of the protein L to bind IgG and make an IgG-linked sepharose column. This would exploit the binding capacity of the protein L, while enabling any other proteins from the bacterial cell lysate to be washed away using copious amounts of buffer. It would also test the protein for ability to bind IgG, while the His tag, although also suitable for purification, would not test this function. The sepharose beads used in this process were cyanogen-bromide (CNBr)-linked and were rehydrated and washed well in buffer before linkage to human immunoglobulin (figure 3.2.4.).

Figure 3.2.4. – Immunoglobulin-linkage to sepharose beads for purification of protein L.

This figure shows an acrylamide gel (A) with immunoglobulin (~120kDa) solution before and after exposure to sepharose beads, and a bar chart (B) plotting the concentration changes before and after exposure to the beads. The pre-bead sample of immunoglobulin was highly concentrated and contained purified human immunoglobulin at a concentration of 40mg/ml - 10 μ l of this solution (400 μ g) was loaded onto the gel. The decrease caused by the beads binding immunoglobulin in solution is clearly seen in a right-hand lane. It was found to contain 52 μ g/ml (column labelled post-beads), compared to a 1mg/ml start solution (column labelled pre-beads). The start solution was diluted 1:40 to enable measurement by optical density).



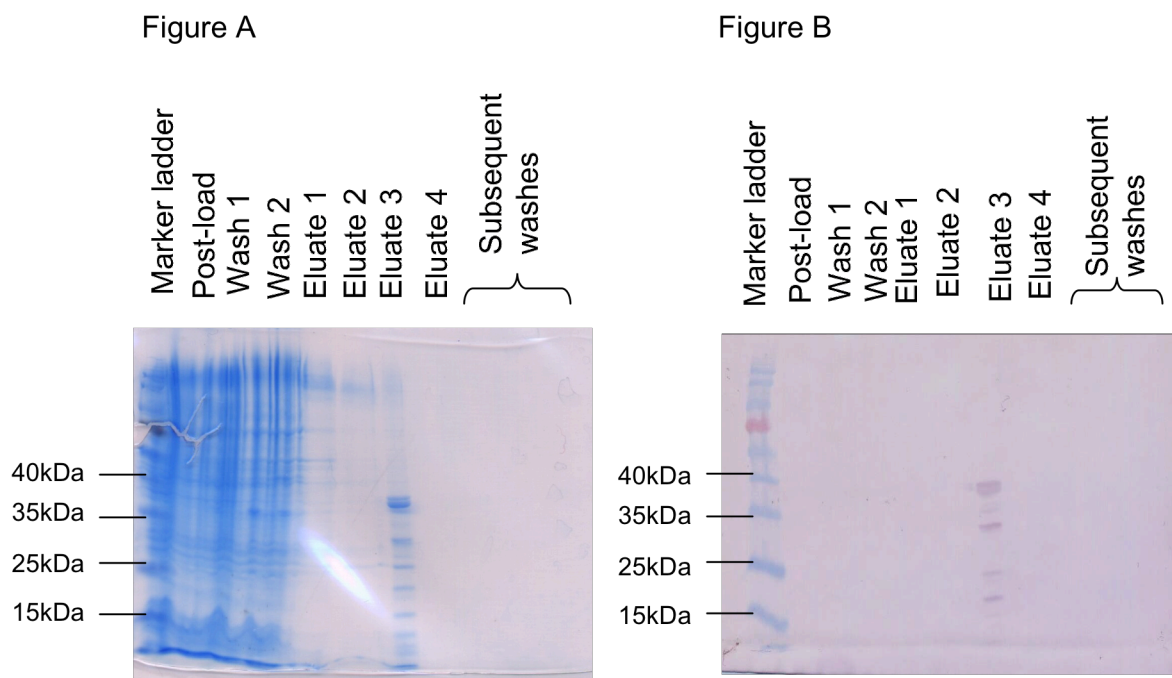
Aliquots of the immunoglobulin solution were run on an acrylamide gel before and after incubation with the beads (figure 3.2.4A) and the protein content of the solution was measured before and after exposure to the beads by optical density at 280nm (figure 3.2.4B). Prior to exposure to the beads, the immunoglobulin solution was 40mg/ml (further diluted to 1mg/ml for O.D. measurement), while after exposure to beads, the concentration of the immunoglobulin solution was 0.052mg/ml. The concentration of antibody attached to the beads was therefore 39.948mg Ig/ml beads.

The bacterial lysate was applied to the column and incubated without any flow through for 1 hour prior to washing with PBS with at least 3 column volumes (approx 75 ml). The protein L was eluted using a pH 2 glycine wash and concentrated by ethanol precipitation overnight (Chapter 2). Figure 3.2.5. shows the coomassie stained acrylamide gel (figure A) and Western blot (figure B) of the protein solution before and after the purification step. The Western blot shows no protein L in the post-load or washes (indicating that all the available protein L has bound to the IgG-linked sepharose) and substantial quantities in the eluates.

On both the coomassie-stained gel (figure 3.2.5A) and the Western blot (figure 3.2.5B), there are multiple bands. The complete protein L insert should be approximately 40kDa, but as seen on the Western blot, the bands correspond to approximately 40kDa, 30kDa, 25kDa and 20kDa. This suggests that the identical repeats within the protein L B1-B4 sequence causes misreading by the RNA polymerase and enables truncated forms of the protein to be produced.

Figure 3.2.5. – Purification of protein L from lysate using IgG-linked beads.

This figure illustrates the various stages of the purification process using the column made from IgG-linked sepharose beads. Figure A shows the coomassie stained gel, while figure B shows a Western blot (probed with anti-HSV antibody) of the different stages of the protein L purification processes. From left to right on each gel: marker ladder, post load, two wash steps, four elution steps, then subsequent washing steps to refresh the column (elution buffer, then more wash buffer). As can be seen, the protein L is eluted in the third elution step. It also elutes at various sizes, this is due to production of the protein with different amounts of the binding domains as the sequence is identical for each domain.



Another explanation for the multiple bands could be that some other molecule within the bacterial lysate also bound to the IgG immobilised within the column, but the Western blot shown was probed with antibody directed against the HSV tag on the protein, making it unlikely that the additional bands are due to contamination.

3.2.3. – Determination of protein L binding to DR15 B cells

Having produced and purified the protein L, it was important to determine whether the protein did what had been previously described in the literature, namely, bind to surface immunoglobulin on B cells. In particular, whether it bound Epstein-Barr virus (EBV)-transformed B cell lines that express the HLA-DR15 molecule was tested. Initial experiments binding the purified protein L to B cells and probing binding with anti-HSV, detected by flow cytometry proved problematic. Despite blocking between antibody steps, the protein L bound the kappa fraction of the detection antibodies and generated a lot of false positive data. This was a particular problem with murine antibodies, as the vast majority of the IgG contain κ light chains (95%) (193), so it was decided to directly conjugate the protein L with FITC.

While the FITC-conjugated protein L was relatively easy to produce (Chapter 2), maintaining the protein in solution was more difficult. The conjugated protein L precipitated out of solution more easily than unlabelled protein L. However, provided labelling and usage were within 2 weeks and storage was in the dark at 4°C, the same batch of protein L could be used for flow cytometry experiments (figure 3.2.6.) and allowed consistency between experiments and the resultant data.

In addition, the protein L-FITC was tested on a mixed population of human peripheral blood mononuclear cells (figure 3.2.7.). This work found that 0.36% of

the cells were positive for both CD19 and Ig κ (figure 3.2.7B), compared with unstained cells (figure 3.2.7A). This is approximately 20% of the total CD19 positive population (1.53%), so lower than the expected 50-60% Ig κ more commonly seen in human cell populations (193). A further set of samples from the same cell population found that 8.68% of the cell population was double-labelled for the protein L and CD19 (figure 3.2.7C). As the CD19-PE antibody was monoclonal and generated in mouse, it has been characterised as IgG₁ with κ light chain, therefore suggesting that the protein L bound to the B cells also bound anti-CD19-PE giving false positive data. However, the value for staining CD19 alone (1.53%) is very low so this data should be approached with caution. These data suggest that the anti-CD19 antibody was defective. These data also emphasise a potential problem with using protein L as a fusion partner for α 3(IV)NC1 or peptides derived from the α 3(IV)NC1 sequence. Since the protein L is ambidextrous, it can bind Ig κ on either side. If one side of the protein L binds Ig κ on B cells, there is still another binding site capable of binding Ig κ on neighbouring B cells (unlikely due to spatial constraints) or in the surrounding solution. Care must therefore be taken that no additional antibody is within the cell suspension to ensure that reactions are due to α 3(IV)NC1 or α 3(IV)NC1 peptides, rather than any other antibody simultaneously binding to the protein L and being internalised by the B cell.

Protein L is supposed to be specific for Ig κ but the flow cytometry data shown in figure 3.2.6. suggested that 74.33% of the cells tested bound the protein L.

Figure 3.2.6. – Flow cytometry analysis of protein L-FITC binding DR15 positive B cells.

This data shows the binding of protein L-FITC to an EBV-transformed DR15 positive B cell line (CRC013). Figure A shows the forward and side scatter plot for the cell line with the gated cell data for unlabelled DR15 positive B cells showing no fluorescent labelling in figure B. Figure C shows the B cells double-stained with CD19-PE (a B cell marker molecule, Y axis) and protein L-FITC (X-axis). As is shown, 74.33% of the cells counted were stained with both the CD19 and protein L-FITC.

Figure A

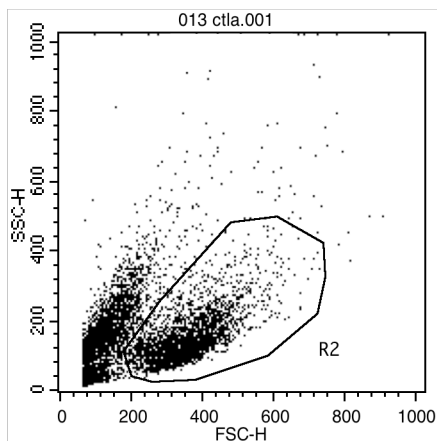


Figure B

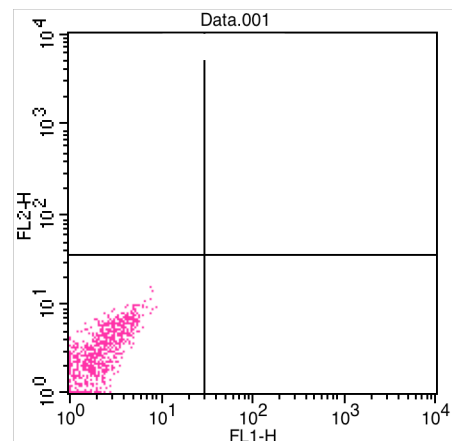


Figure C

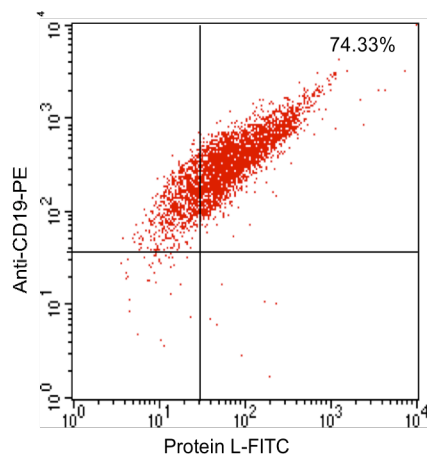


Figure 3.2.7. – Flow cytometry analysis of Igκ and protein L-FITC binding CD19 positive B cells.

This figure illustrates the data obtained from labelling human peripheral blood mononuclear cells with CD19-PE and Igκ-FITC or protein L (unlabelled protein L, so secondary, anti-HSV, and tertiary, anti-mouse-FITC, antibodies used for counter staining). Figure A shows the unlabelled cell population and gate, figure B shows the gated cell population dual labelled with anti-Igκ-FITC and anti-CD19-PE to give a double positive population of 0.36% (total CD19 positive population of 1.53%). Figure C shows the data obtained from double labelling with protein L-FITC and CD19-PE. This shows a double positive population of 8.68%, suggesting that the protein L also binds mouse anti-CD19-PE antibodies, which are likely to be Igκ and therefore generating false positive data.

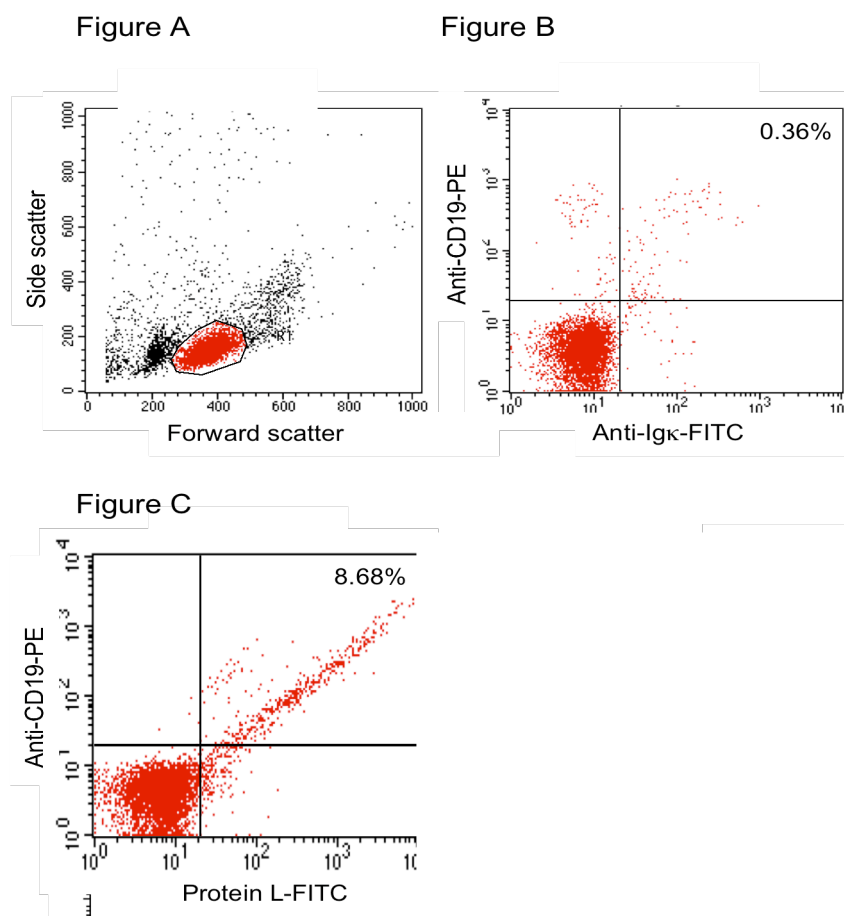


Figure 3.2.8. – FACS plots showing Ig κ and Ig λ staining on DR15 positive B cells.

These plots illustrate the proportions of Ig κ expressing B cells compared with Ig λ expressing B cells from a B cell line expressing DR15 molecules. Figure A shows unstained cells (isotypic controls for the antibodies gave a similar plot, data not shown). There is a 96.28% population shift when staining the B cells with anti-Ig κ -FITC (figure B) and a 22.06% population shift when staining the cells with anti-Ig λ -FITC (figure C).

Figure A

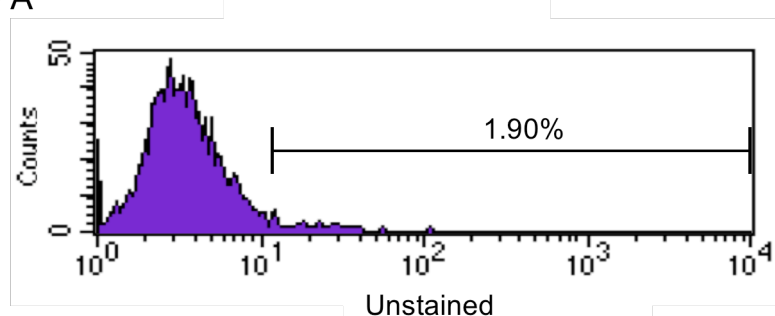


Figure B

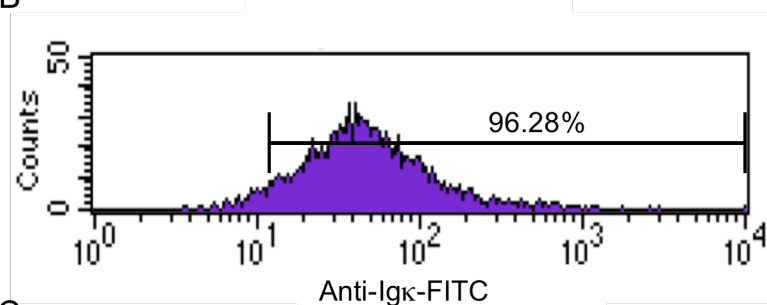
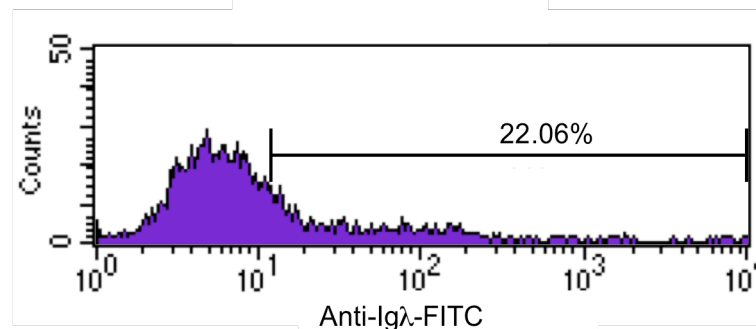


Figure C



Therefore, it was decided to test the cell line used, by flow cytometry, for Ig κ and Ig λ surface expression (figure 3.2.8.). This was surprisingly inconclusive, as the data suggests that 96.28% of the B cells expressed Ig κ , but that 22.06% expressed Ig λ (dual labelling was not possible as both antibodies were directly conjugated with FITC). This suggests that some B cells were expressing both κ and λ chains, so implies cross-reactivity of the Ig κ antibody as normal B cells only express one of either Ig κ or Ig λ . However, these B cells are EBV-transformed so cannot be described as normal. Furthermore, B cells in some cancers do express both Ig κ and Ig λ (194). This finding is emphasised by the data being consistent at two different dilutions of the anti-Ig κ antibody (1:10 and 1:100).

It could be argued that some sample populations may have included 100% of Ig κ -expressing B cells. However, each label was investigated in triplicate, and at a minimum of two dilutions, so fluctuations in population proportions should be expected and should show levels more approximate to normal reported expression of Ig κ - approximately 60% Ig κ , 40% Ig λ (193). In mice, B cells express 95% Ig κ and 5% Ig λ (195), but since the EBV transformed B cells were derived from human B cells, the former proportions would be expected. These data suggest that the protein L is not as specific for Ig κ as has been suggested (192, 196, 197), since the flow cytometry data using the Protein L-FITC suggested that 74.33% cells used throughout these experiments bound the protein L (figure 3.2.6B).

3.2.4. – Confocal microscopy of protein L binding to DR15 B cells

Confocal microscopy was used as an alternative method to determine whether the protein L-FITC bound to B cells. The images obtained by confocal microscopy negate the binding on such a high percentage of cells, as there were clearly more than the remaining percentage (3.72% seen by flow cytometry) unstained in various fields of the microscope slide (data not shown). These cells were stained with 1.5µg protein L-FITC for 15, 30 or 60 minutes (figures 3.2.9B-D) and counterstained with DAPI (a nuclear stain, shown without the protein L-FITC, in figure 3.2.9A). All of the time points showed some binding of the protein L-FITC, but by 60 minutes, it was clearly intracellular (white arrow, figure 3.2.9D), although it remained in the cytoplasm. It was not possible to determine exactly where the protein L-FITC localised to, as there were some problems with the FITC bleaching under sustained excitation. The images shown are composites of multiple images taken through a Z-stack, but multiple Z-stacks on multiple fields proved difficult owing to bleaching.

An alternative explanation for the excessive binding of protein L to the B cell line, other than cross-reactivity and non-specific binding, could be that the protein L-FITC preparation was not sufficiently clean of excess, unconjugated FITC molecules. This would mean that the flow cytometry data also shows cells that have bound FITC alone, rather than protein L-conjugated FITC. This is possible as the conditions for FITC binding to a purified protein are quite specific and needs to be performed in the absence of amine groups. The FITC will preferentially bind amine groups over alternatives, thus reducing the labelling on the requisite protein.

Figure 3.2.9. – Confocal images of Protein L-FITC binding DR15 positive B cells, double stained with DAPI.

These composite images show DR15 positive B cells double stained with DAPI (a nuclear stain, blue) and protein L-FITC (green). Each cell population was incubated with 1.5 μ g protein L-FITC for various time points; figure A shows unstained cells (DAPI only), then stained for 15 minutes (figure B), 30 minutes (figure C) and 60 minutes (figure D). As can be seen, staining does not occur on all cells, but the protein L-FITC does appear to be internalised into the cells to which binding is seen (figure D, white arrow) and occurs with relative speed as binding is observed after 15 minutes of incubation (white circle, figure B).

Figure A - DAPI

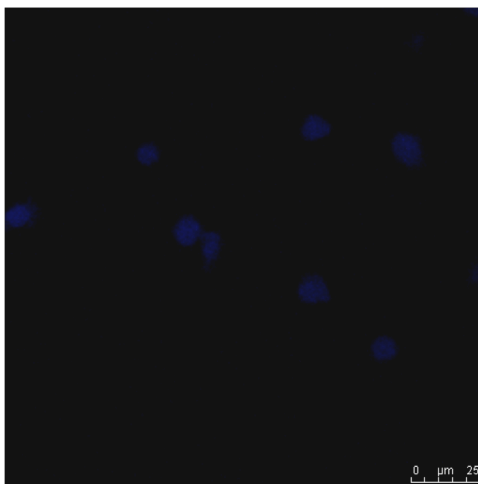


Figure B- Protein L, 15min

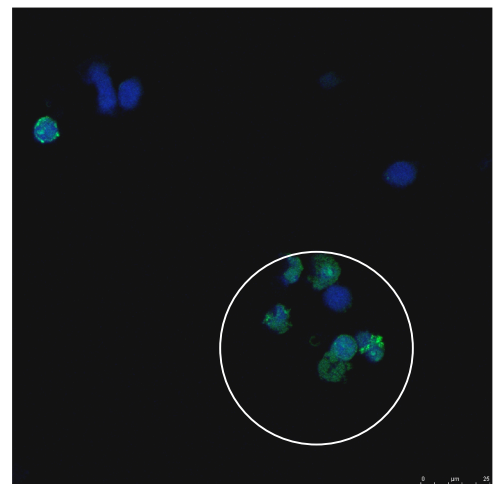


Figure C - Protein L, 30min

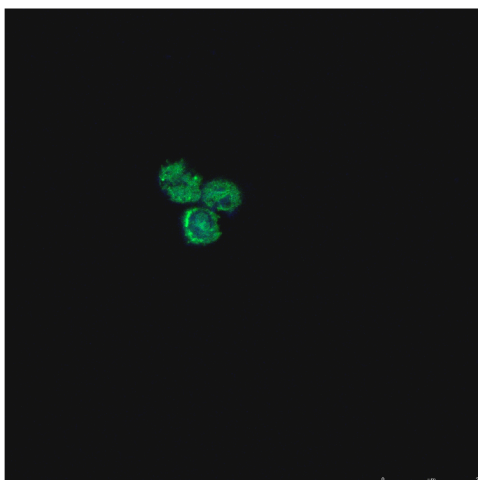
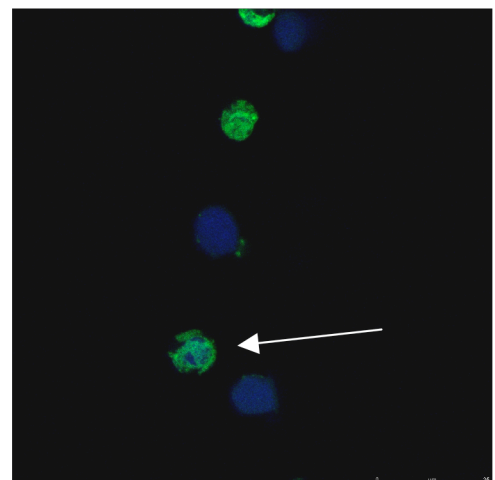


Figure D - Protein L, 60min



The active group is also destroyed by water so is never present in FITC-protein conjugates. It does mean that the FITC-labelled protein is hydrophobic and can “stick” without being actively bound, therefore generating false positive data. The presence of excess unbound FITC within the protein L-FITC solution was minimised by utilising molecular weight spin-columns with a cut off of 10 000Da – these ensured that the majority of unbound FITC was centrifuged away from the final protein L-FITC solution used for the experiments described.

3.3. - Discussion

Having shown that protein L produced was capable of binding to DR15-expressing B cells, the next stage of this project was to produce protein-L conjugated to a specific peptide and show whether it improves antigen presentation by increasing the amount of IL-2 produced by peptide specific hybridomas, compared to hybridomas stimulated with B cells and peptide without the protein L.

Briefly, the $\alpha 3$ sequences focussed on for addition to the protein L were the $\alpha 3(\text{IV})\text{NC1}$ 136-150 peptide, to which 100% Goodpasture patients generate an immune response and to which 100% DR15 A3v $\alpha 3\text{KO}$ mice also generate an immune response. This peptide was also suitable for use in these constructs as there was already a hybridoma responsive to this peptide available. The second peptide considered for addition to the fusion protein was the $\alpha 3(\text{IV})\text{NC1}$ 64-90 peptide, a peptide with known immunological activity in Goodpasture patients, but which is also known to be cleaved almost immediately within the lysosome.

The $\alpha 3(\text{IV})\text{NC1}$ 136-150 fragment is a region of the $\alpha 3(\text{IV})\text{NC1}$ sequence to which 100% patients with Goodpasture's Disease have a measurable proliferative T cell response compared to HLA-DR matched control samples. In this thesis, it is also a region to which the transgenic KO mouse model produces an immune response (as discussed in Chapter 6). With this in mind, oligonucleotide sequences were designed to amplify the $\alpha 3(\text{IV})\text{NC1}$ 136-150 sequence of the $\alpha 3(\text{IV})\text{NC1}$ with specific restriction sites on either end. This fragment could then be ligated into the protein L construct plasmid, resulting in production of a fusion protein complex called protein L-A3 136-150. The primer and relevant PCR products were produced, but ligations into the protein L plasmid failed and test digests for the WISL sequence in conjunction with the protein L sequence proved negative (data not shown).

Other uses for protein L include it being used as part of a hybrid protein, and have been projected to improve extraction of immunoglobulins in general (198, 199). Many antibodies are purified using protein G, but this has a more limited binding capacity than protein L. In particular, protein L could be very useful in the purification of murine antibodies from serum since these are mostly Ig κ (~95%). A hybrid protein between protein G and protein L could therefore dramatically improve the rate of recovery of antibody from serum samples (199). A hybrid protein consisting of protein L and protein A would have similar applications. Protein A is also used in the purification of immunoglobulins and binds IgG Fc regions, so a hybrid protein could also improve the recovery of antibodies from a given serum sample (198).

Overall, it appears that protein L is capable of binding to B cells and that it could be used as a potential fusion protein with which to improve uptake into B cells.

However, more work will need to be done in order to understand the mechanism of uptake and the route within the cell to ensure processing pathways are not different from the normal mechanisms. This could include labelling the protein L with a different fluorescent molecular to reduce bleaching, combined with time-lapse confocal at higher resolution to track the progress of the protein L into cells, as well as continuing the projected work attaching an $\alpha 3$ peptide to the protein and determining whether peptide-specific hybridomas respond. Overall, protein L would enable the direct comparison of immune responses *in vitro*, and would increase the chances of a specific antigen being taken up into the cells, thereby improving the levels of different peptides presented. Protein L could be used to improve the presentation of more rare peptides with lower capacity to induce an immune response on the B cell surface. These peptides may have a major role in the induction of tolerance during T cell repertoire development, so protein L could enhance understanding of central tolerance and its role in the development of autoimmune disease.

To summarise, protein L appears to recognise Igk, to specifically bind B cells, and to be internalised by B cells upon binding. This information suggests that protein L could be an efficient fusion partner when studying antigen presentation and could be exploited to study and enhance B cell uptake of specific fragments of antigen. Unfortunately, time constraints meant that it was not possible to link protein L to $\alpha 3(\text{IV})\text{NC1}$ or fragments of the $\alpha 3(\text{IV})\text{NC1}$ protein during this project. However, the constructs were stored such that additional work could be undertaken to further explore potential uses for this protein as a means by which to improve antigen uptake into B cells.

Chapter 4 – Development of transgenic mouse line

4.1. - Introduction

The purpose of experiments described within this chapter was to develop a transgenic mouse expressing the human MHC class II molecule HLA-DR15, which also expressed or did not express the Goodpasture antigen, $\alpha 3(\text{IV})\text{NC1}$. These mice were used to study immune responses and antigen presentation in the presence and absence of endogenous $\alpha 3(\text{IV})\text{NC1}$, and therefore in the presence or absence of tolerance.

Before creating a transgenic mouse line, it is important to consider what alternatives are available and how much will actually be gained from using mice. Mice differ from humans in physiology, and some different biochemistry. This means that while some parallels can be drawn, particularly in the use of transgenic animals where the animal has to some extent been “humanised”, it is only really possible to investigate mechanisms and discuss possibilities rather than certainties (200, 201). The main reason for development of animal models of disease is in order to identify processes that occur before and during disease to determine mechanistic features with a view to either inhibiting or preventing disease progression. There are two different types of disease model – those that develop disease spontaneously, for example non-obese diabetic (NOD) mice (202, 203), and

those in which disease is induced, usually by a drug administered in the diet, for example streptozocin induced diabetic mice (204, 205).

In this project, the model developed had known MHC class II expression restricted to HLA DR15, enabling control of when the immune responses to a specific antigen were initiated. It would therefore be possible to investigate specific features of the immune response in a way that is not possible with human Goodpasture patients or normal HLA-matched volunteers. In particular, it would be unethical to immunise humans with an antigen that could potentially cause autoimmunity and renal failure. The experimental protocol would be very different and would utilise blood samples from untreated Goodpasture patients and HLA-matched controls, but while work has been published using samples from untreated human Goodpasture patients to study their immune responses (206, 207), it is difficult to gain sufficient blood and sufficient numbers of patients to make this work statistically significant. This is mostly due to the scarcity of new patients and the overall rarity of Goodpasture's disease, but also because newly presenting Goodpasture patients are very ill and withholding treatment for the sake of additional blood samples, if needed, is unethical.

Although some aspects of this disease have previously been studied (41), in particular experiments to determine cellular responses to specific fragments of the Goodpasture antigen, it has not been possible to investigate the immune responses in the presence and absence of tolerance. Another disorder involving the collagen IV chain is Alport's disease. Patients with Alport's diseases have impaired renal function as a result of lacking or expressing mutated the $\alpha 5(\text{IV})$ collagen chain, and similar disease aetiologies have also been reported to occur with alterations in the $\alpha 3$

or $\alpha 4$ chains (208). Treatment for Alport's disease can include transplant, and these patient's can develop Goodpasture-like autoimmunity as a result of not developing tolerance to the normal $\alpha(IV)$ chain (209-211). This disorder could be considered a viable human comparison regarding immune responses in the presence and absence of tolerance. However, Alport's disease is also very rare; it is a known X-linked genetic disorder rendering it dissimilar to Goodpasture's disease; and sufficient samples to enable study of immune responses would be extremely difficult to obtain. Despite these limitations, this a potential route for investigating immune responses to specific autoantigen given time and sufficient samples.

It has also been suggested that environmental factors have a role in the onset of Goodpasture's disease (212, 213). It is far easier to control the surrounding environmental influences on mice than it would be to restrict the environment in humans. In particular, approximately 10% of patients used to present with pulmonary haemorrhage, but this number has reduced over time (closer to 5%) and there is a positive correlation between pulmonary involvement and smoking ($p > 0.01$) (213, 214). It was also shown that some of these patients worked with toxic chemicals and the haemorrhage could have been allowed by inhalation of these chemicals (212), which may have then increased the accessibility of the underlying collagen structure and enabled the autoimmune reactions seen in the kidney to also occur within the delicate lung tissue.

The consensus for onset of autoimmunity is that there is a "double hit". It is thought that there is an underlying predisposition for the disease (the first "hit"), at least by HLA type as well as other, less well-described immunological molecules, and then some additional stimulus, a second "hit". For example, in Goodpasture's

disease, it has been shown that patients usually express HLA-DR15. It has also been reported that some kind of trauma within the kidney – surgery, lithotripsy for kidney stones for example - may have had an influence upon the disease development, but these patients are in the minority (215).

Another limiting factor is the availability of blood samples. Unless the patients were untreated, the immunosuppressive therapy involved in treatment of this disease would destroy subsequent experiments studying the specific T cell responses. A mouse model could help overcome this problem. The scarcity of new patients would also be a considerable hindrance to this project as, on average, there is estimated to be fewer than 1 patient per million people per year, although there is limited information about the population incidence and prevalence (216).

In addition to the advantages to using a mouse line rather than human samples, the mouse line allows us to study the influence of tolerance on the immune response, as animals can be bred both with and without endogenous $\alpha 3$. It would be logical to assume that mice that do not express the $\alpha 3(\text{IV})\text{NC1}$ domain, when immunised with exogenous $\alpha 3(\text{IV})\text{NC1}$, would treat the protein as ‘foreign’ and mount an immune response to it. This response could then be tested to determine which peptide fragments of the $\alpha 3(\text{IV})\text{NC1}$ domain produce the greatest immune response. By comparison, the mice that express the $\alpha 3(\text{IV})\text{NC1}$ protein would be tolerised to the protein so it would be more difficult to induce an immune response to any part of the protein. In these mice, it is possible that any immune responses are to so-called cryptic epitopes and could help determine where the cryptic epitopes lie, and any specific similarities between these peptides.

These factors considered, a transgenic mouse line was the most feasible option for studying the immune responses to the Goodpasture antigen. A major characteristic of Goodpasture patients, is that 90% express the human MHC class II molecule HLA-DR15 (as discussed in Chapter 1), while the target antigen, the $\alpha 3$ chain of type IV collagen ($\alpha 3(\text{IV})\text{NC1}$), has also been well characterised. Development of a mouse line with a CD4 immune response restricted to presentation of peptides only by HLA-DR15, both with and without endogenous $\alpha 3$, would enable determination of immune response by antigenic fragments. These responses could then be studied to determine which antigenic peptides produce an immune response both in the presence and absence of endogenous $\alpha 3$ and therefore to study the influence of self upon responsiveness to self-antigen.

4.2. – Results

4.2.1. – Cross-breeding to generate the DR15 A3v transgenic mouse line

Two mouse lines were available for this project: 4A3 carries a mutation that is $\alpha 3\text{KO}$ ($\alpha 3(\text{IV})$ knock-out): the progeny can be wild type ($\alpha 3\text{-WT}$), heterozygous ($\alpha 3\text{-Het}$) or knock-out ($\alpha 3\text{-KO}$) for the $\alpha 3$ chain. This line was developed by Miner *et al.* in St Louis, Missouri (174) and has been used to investigate the influence of genetic deletion of a component of the $\alpha 3\text{-}\alpha 5$ collagen IV network analogous to Alport's disease phenotype in which the $\alpha 5$ chain is absent. The second line used was the DR15 mouse line, which is knocked out for mouse class II molecules, but transgenic for the human genes that encode the HLA-DR15 molecule, HLA-

DRB1*1501 and HLA-DRA1*0101. The original mouse class II KO was produced by Cosgrove *et al.* (176), with the DR15 double transgene insertion added by Prof Altmann's group in London (175). It was therefore decided to cross-breed these lines and generate a mouse line with a restricted MHC class II repertoire. There was also the potential for an increased propensity towards the development of glomerulonephritis as a side effect of the cross-breeding.

The breeding process was more difficult to implement than implied above, as both lines are relatively complex to breed. The 4A3 line (on a 129sv background) (174) cannot breed from A3-KO female animals, as they die during pregnancy or immediately postnatal. It is thought that the kidneys of the A3-KO animals are unable to cope with the additional filtration required and pressures of pregnancy and enter acute renal failure (personal observations). The choice of breeding animals to produce the required genotype and maintain the colony was therefore A3-KO stud males breeding with A3-Het females producing both A3-Het and A3-KOs pups per litter. The A3-KO mice do have a tendency to be smaller than their littermates, but this is by no means a reliable way to identify the A3-KO mice from the others.

The DR15 line (on a C57BL/6 x CBA background) (175, 217), as mentioned above, is also complicated. The mouse class II molecule, when knocked out within an animal by insertion of a neomycin cassette within the gene, appears to have no effect on the breeding capacity of that animal, so class II-KO males can breed with class II-KO females without repercussions. However, the additional level of complexity arises with HLA DR15 expression. The HLA DR15 molecule is expressed as 2 chains, α and β , both of which have to be expressed in order for the

Figure 4.2.1. – Punnet square showing the initial cross to produce DR15 A3v line.

The cross was set up with an F₀ male $\alpha 3$ KO (vertical axis) bred with a F₀ female mouse class II KO, double transgene human MHC class II insert (horizontal axis). The second from right and right hand columns show the frequencies of the resultant female and male genotypes. The overall yield is calculated by multiplying the frequencies together. The **desired** female genotype was mouse class II het, HLA-DRA positive, HLA-DRB positive, $\alpha 3$ het.

F ₀ ♂ \ F ₀ ♀	F ₁ cross genotypes at all four loci				Yield (of desired females)	Yield (of males)
	MHC IAB ^{-/-}	HLA DRA1*0101 Tg/0	HLA DRB1*1501 Tg/0	COL IV A3 ^{+/+} (mouse)		
MHC IAB ^{+/+}	<u>MHC IAB^{+/-}</u>				100%	100%
HLA DRA1*0101 0/0		<u>HLA DRA1*0101 Tg/0</u> or HLA DRA1*0101 0/0			50%†	
HLA DRB1*1501 0/0			<u>HLA DRB1*1501 Tg/0</u> or HLA DRB1*1501 0/0		50%†	
COL IV A3 ^{-/-} (mouse)				<u>COL IV A3^{+/-}</u>	100%	100%
				Overall yield:	25%	100%

† Only female offspring carry the MHC class II A or B chain transgenes.

molecule to be produced and function on the cell surface. In the cross, hereafter called DR15 A3v (DR15 expressing, $\alpha 3$ variable), both of these breeding issues have to be taken into consideration.

The initial cross was such that male 4A3 mice KO for $\alpha 3$ were set up in breeding pairs with females KO for mouse class II, but positive for the DR15 genes. The resultant F₁ genotypes and frequencies are shown in figure 4.2.1.

The subsequent F₁ breeding pairs used male IAB^{+/-} COLIVA3^{+/-} mice crossed with female IAB^{+/-} DRA^{Tg/-} DRB^{Tg/-} COLIVA3^{+/-} and the resultant F₂ genotypes and frequencies are shown in figure 4.2.2. As seen in these figures, the genes affected are listed as IAB, DR15 A/B double transgene, COLIVA3. The shorthand developed to describe this line lists the genes in this order and as KO, Het or WT for the IAB and the COLIVA3 genes, with the DR15 transgene described as -/- (not present) or A/B (both chains present). Therefore, all the males used in breeding when the colony was established were either KO -/- KO (IAB KO, DR15 negative, COLIVA3 KO) or KO -/- Het (COLIVA3 Het if COLIVA3 WT offspring were required for use as controls) and females were KO A/B Het (IAB KO, DR15 α/β positive, COLIVA3 Het). Throughout this thesis, this is the annotation used to designate the animals used for any experiments. Additionally, only females were shown to carry the HLA $\alpha\beta$ transgene, so all experimental mice were female.

All subsequent breeding to generate a colony were male IAB^{-/-} COLIVA3^{-/-} bred with female IAB^{-/-} DRA^{Tg/-} DRB^{Tg/-} COLIVA3^{+/-} to give a frequency of 50% total male offspring IAB^{-/-} COLIVA3^{-/-} and 25% total female offspring positive for the DRAB double transgene. Of these females, 50% are COLIVA3^{+/-} and 50% are COLIVA3^{-/-} to give an overall frequency of 12.5% total females (Figure 4.2.3.).

Figure 4.2.2. – Punnet square showing the F₁ cross, the resultant genotypes and frequencies.

This diagram illustrates the second cross of F₁ offspring: F₁ male, mouse class II het, HLA-DRA –ve, HLA-DRB –ve, α3 het (vertical axis) x F₁ female, mouse class II het, HLA-DRA +ve, HLA-DRB +ve, α3 het (horizontal axis). The calculated frequencies of females (**desired**, MHC IAB^{-/-}, HLA DRAB, COLIVA3^{+/-}) and males in the second from right and right hand columns. Again, overall yield was calculated by multiplying the frequency of each genotype (ms class II x MHC class II x α3).

F ₁ ♀ F ₁ ♂	F ₂ cross genotypes at all four loci				Yield (of desired females)	Yield (of males KO - /- KO)
	MHC IAB ^{+/-}	HLA DRA1*0101 Tg/0	HLA DRB1*1501 Tg/0	COL IV A3 ^{+/-} (mouse)		
MHC IAB ^{+/-}	MHC IAB +/+ MHC IAB +/- <u>MHC IAB</u> <u>-/-</u>				25%	25%
HLA DRA1*0101 0/0		<u>HLA DRA1*0101</u> ^{Tg/0} or HLA DRA1*0101 ^{0/0}			50%†	
HLA DRB1*1501 0/0			<u>HLA DRB1*1501</u> ^{Tg/0} or HLA DRB1*1501 ^{0/0}		50%†	
COL IV A3 ^{+/-} (mouse)				COLIVA3 ^{+/-} <u>COLIVA3</u> ^{+/-} COLIVA3 ^{-/-}	50%	25%
				Overall yield:	3.125%	6.25%

† Only female offspring carry the MHC class II A or B chain transgenes.

Figure 4.2.3. – Punnet square illustrating all subsequent breeding pairs and possible genotypes to maintain the new colony.

This diagram illustrates the third cross of F₂ offspring: F₂ male mouse class II KO, DRAB –ve, α3 KO (vertical axis) x F₁ female mouse class II KO, MHC class II AB positive, α3 het (horizontal axis), with the calculated frequencies of **desired** females (mouse class II KO, HLA-DRA +ve, HLA-DRB +ve, α3 het (breeding) or KO (experimental)) and males in the second from right and right hand columns. Again, overall yield was calculated by multiplying the frequency of each genotype (mouse class II x MHC class II x α3).

F ₂ ♀ F ₂ ♂	F ₃ cross genotypes at all four loci				Yield (of desired females)	Yield (of males KO - /- KO)
	MHC IAB ^{-/-}	HLA DRA1*0101 Tg/0	HLA DRB1*1501 Tg/0	COL IV A3 ^{+/-} (mouse)		
MHC IAB ^{-/-}	<u>MHC IAB</u> ^{-/-}				100%	100%
HLA DRA1*0101 0/0		<u>HLA DRA1*0101</u> ^{Tg/0} or HLA DRA1*0101 ^{0/0}			50%†	
HLA DRB1*1501 0/0			<u>HLA DRB1*1501</u> ^{Tg/0} or HLA DRB1*1501 ^{0/0}		50%†	
COL IV A3 ^{-/-} (mouse)				<u>COLIVA3^{+/-}</u> <u>COLIVA3^{-/-}</u>	50%	50%
				Overall yield:	12.5%	50%

† Only female offspring carry the MHC class II A or B chain transgenes.

4.2.2. – Genotyping reactions

In developing this transgenic mouse line, a number of obstacles had to be overcome, the major regarding appropriate breeding pairs in order to maintain the cross. In setting up the optimal breeding pairs to maintain the cross and generate healthy pups, it was necessary to breed from COLIVA3 heterozygous females and therefore detailed genotyping of each animal was required (results figures 4.2.1.-3.). The primers and conditions used for each PCR reaction are described in chapter 2, but additional details are given below.

For identifying whether animals were knocked out for the mouse class II gene (figure 4.2.4.) the following primers were designed:

ABOL2: 5'-CAC CAA CGG GAC GCA GCG CAT AC-3'

ABOR2: 5'-GGC CCT CGT TCG CTC CAG GAT CT-3'

These primers bind the mouse class II gene (this strain only carries the IAB gene and not the IEB mouse class II gene) and result in a wild type fragment of 169 base pairs (bp) if the gene is intact. If the gene has successfully been disrupted, a cDNA cassette encoding neomycin has been inserted into the mouse class II gene. This disruption gives a fragment that includes the neomycin as well as the IAB gene fragment and is 1000bp (figure 4.2.4A). In addition, a second PCR reaction was carried out to check the presence of the neo cassette and used the ABOL2 primer with an additional primer:

Neo: 5'-GAG GAT CTC GTC GTG ACC CA-3'

The Neo primer binds within the neomycin cassette and, when used in a pair with the ABOL2 primer, results in the amplification of a fragment 340bp in length (figure 4.2.4B).

The insertion of the MHC class II transgene was also tested by PCR reactions. The primer pairs each recognise either the HLA DRA1*0101 gene or the HLA DRB1*1501 gene so two PCR reactions were performed to determine the HLA status. The primers were designed as follows:

TgDRAL: 5'-CTC CAA GCC CTC TCC CAG AG-3'

TgDRAR: 5'-ATG TGC CTT ACA GAG GCC CC-3'

TgDRBR: 5'-CTG CAC TGT GAA GCT CTC-3'

TgDRBL: 5'-TTC AAT GGG ACG GAG CGG GTG-3'

In combination, the TgDRAL and TgDRAR result in the amplification of a fragment 124bp in length, while the TgDRBL and TgDRBR amplify a fragment approximately 190bp, as shown in figure 4.2.5.

The final set of PCR reactions were to determine the $\alpha 3$ status of the mice. In this case the mouse COLIVA3 gene was also disrupted by insertion of a neo cassette and could be detected by targeted PCR specific for this cassette. The primers are shown below:

4A3 KOWTL: 5'-ACG ACC TTT GTT AAA CTA GAA GAA GTC-3'

4A3 WTR: 5'-TTC CCC TGT CAC CAG GAT TTC CC-3'

4A3 KOR: 5'-TGC TAA AGC GCA TGC TCC AGA CTG C-3'

These primers were designed such that the 4A3 KOWTL primer could be used with both the 4A3 WTR and 4A3 KOR primers and produces different sized bands in

Figure 4.2.4. - Mouse class II PCR reaction.

Gel showing results from PCR reactions for IAB (WT) and for the neomycin cassette inserted into the IAB gene to disrupt and prevent transcription of the gene (KO) (figure A). From left, Hae marker ladder, IAB WT, IAB KO as shown by increased size within the IAB (mouse class II) gene. Also shown are Neo PCR results for the neomycin insert in the IAB gene (figure B). From left, 100bp marker ladder, Neo negative (IAB WT), then Neo positive (IAB KO).

Figure A

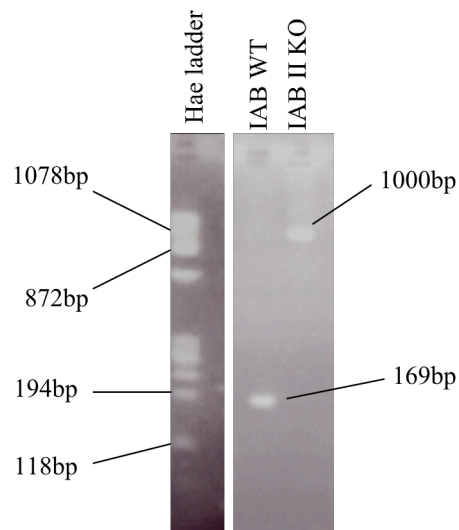


Figure B

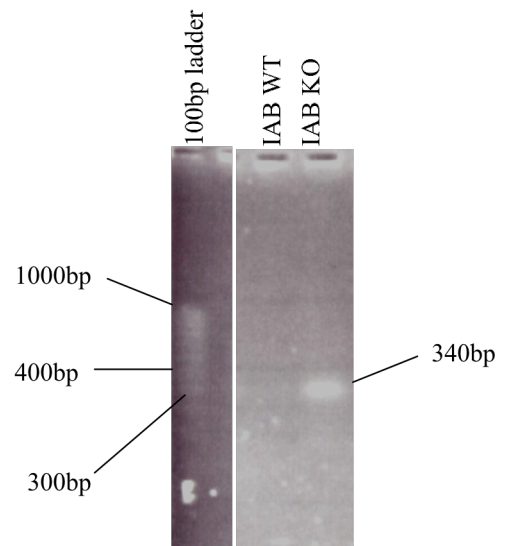


Figure 4.2.5. – MHC class II PCR reaction for genotyping.

Image derived from a gel on DNA samples tested for the MHC class II transgene. From left, Hae marker ladder, DRA chain positive, DRA chain negative, DRB chain positive, DRB chain negative. The DRA positive reaction generates a fragment of approximately 124bp, while the DRB reaction generates a fragment of approximately 190bp . As with gel images shown above, the presence of a band is a positive reaction.

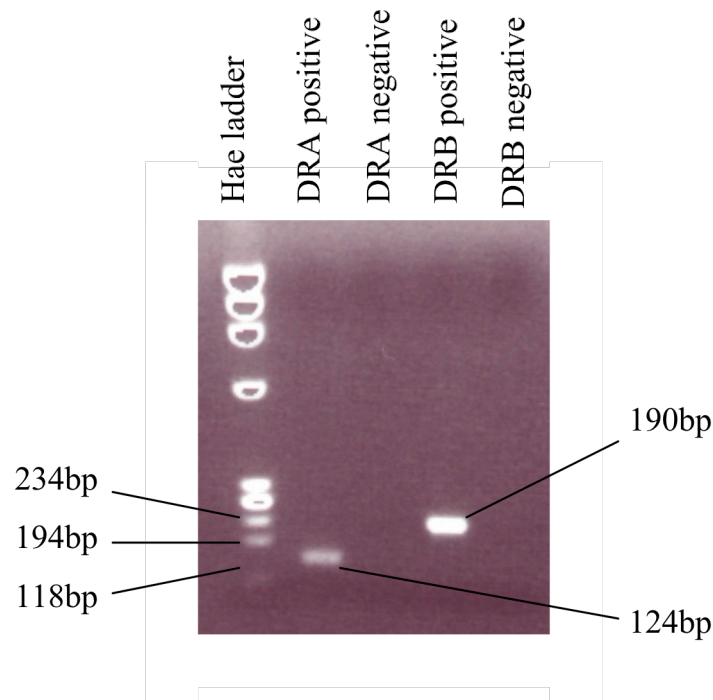
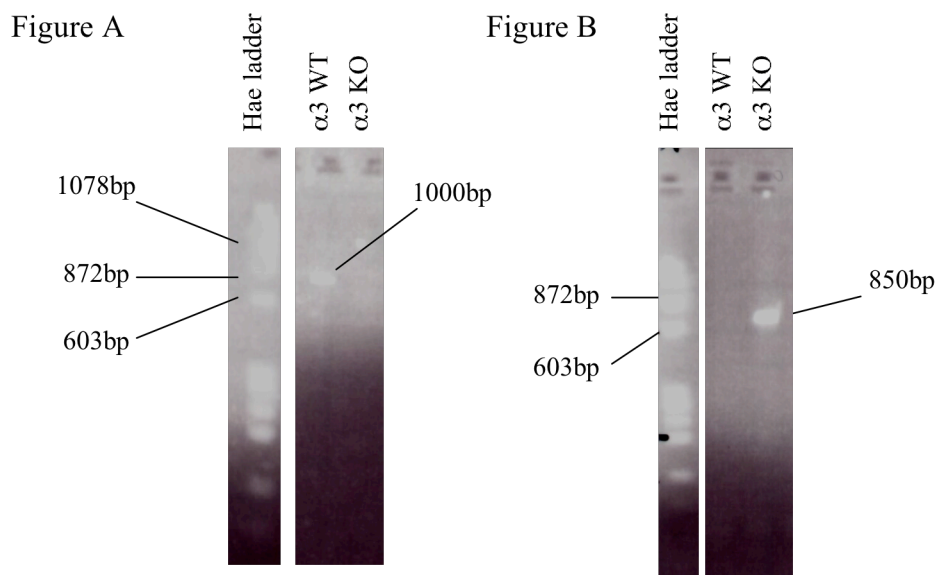


Figure 4.2.6. – PCR genotyping reactions for $\alpha 3$ WT and $\alpha 3$ KO.

Each image shows three lanes of a gel. Lane 1 contains the Hae ladder, lane 2 the $\alpha 3$ wild type DNA sample and lane 3 the $\alpha 3$ knockout DNA sample. Wild-type positive band is approximately 1000bp (figure A), while the KO band is approximately 850bp (figure B). In figure A, the band appears lower than 1000bp, however the entire gel ran faster in lane 2 than 1, 3 than 2 and so on, rendering it difficult to determine exact size. However, presence of the band is sufficient to denote a positive reaction.



each. The WT reaction amplifies a fragment approximately 1000bp in length, while the KO reaction amplifies a fragment approximately 850bp in length (figure 4.2.6.).

4.2.3. – Frequency of genotypes

Since this was a new transgenic crossed, the frequency of occurrence of each genotype derived from a specific breeding pair was investigated and used to maximise chances of obtaining mice that both expressed the MHC class II AB transgene and were knocked out for the $\alpha 3$ gene. Prior knowledge about the effects of the transgenes was taken into consideration for these studies, as the requirements for breeding the various required genotypes were maintained by experienced animal technicians. The data obtained from this study is shown in figure 4.2.7. This figure illustrates the possible genotypes derived from specific breeding genotype pairings, the expected frequencies and the observed frequency of each.

In a study of 200 mice, chosen for the genotype of the parents, two things became very obvious. Firstly, there is a positive skew in favour of producing female mice. Out of the 200 mice studied, 50 were male and 150 were female. While some strains of mouse do have a tendency towards producing more of one sex than the other, this observation is a statistically significant difference ($\chi^2_1 = 50$, $P = 1.2 \times 10^{-5}$). In addition, out of the 50 males born, only 5 genotypes of a possible 8 were actually seen and the majority of those (38/50) were either DRAB^{-/-} COLIVA3^{+/-} (22/50), or DRAB^{-/-} and COLIVA3^{-/-} (16/50). The other genotypes seen were DRA^{Tg/-} COLIVA3^{+/-} (2/50), DRB^{Tg/-} COLIVA3^{+/-} (9/50), and DRB^{Tg/-} COLIVA3^{-/-} (1/50). This suggests that the AB combination of genes does not support the male genotype for a variety of possible reasons, and these are discussed in section 4.3.

By way of comparison, of 150 female mice, 65 were DRAB^{Tg/-} COLIVA3^{+/-} and 13 were DRAB^{Tg/-} COLIVA3^{-/-}. The DRAB^{Tg/-} COLIVA3^{-/-} numbers are reasonably close to the expected frequency of 6.25% per genotype (observed frequency of 6.5%), assuming that each characteristic has a 50% chance of occurrence:

sex 0.5 x A 0.5 x B 0.5 x α3 0.5 = 0.0625, therefore 6.25% frequency of occurrence.

In reality, the range of frequencies and numbers within each genotype were dramatically different, the overall data being influenced by the frequency of the female DRAB^{Tg/-} COLIVA3^{+/-} and, following testing by chi-squared statistics gave a value of $\chi^2_{15} = 347.36$ ($P = 7.5 \times 10^{-65}$), showing that the observed frequency of total genotypes is significantly different from the expected frequency of total genotypes (figure 4.2.7.). Females were born with the HLA DRAB double transgene more frequently than was expected (32.5% when also COLIVA3^{+/-}, a lower frequency 6.5% when COLIVA3^{-/-}), especially when compared to the numbers from males of the same genotypes (0% of either). In both sexes, the HLA DRAB^{-/-}, COLIVA3^{+/-} genotype also occurred more frequently than was anticipated from prior calculations (males 11%, females 16%). The genotype HLA DRAB^{-/-}, COLIVA3^{-/-} also occurred at 1.8 times the frequency in males than it does in females, so, to correct for the skew in favour of females, effectively occurs 5.3 times more frequently in males than females. These considerations had to be taken into account when breeding required genotypes, both for subsequent breeding and to produce the appropriate genotypes for experimental purposes.

Figure 4.2.7. – Schematic of a typical breeding pair of the DR15 A3v line showing offspring genotypes and expected vs observed frequency.

This chart shows the parent genotypes, possible offspring genotypes, the calculated expected frequency of occurrence, and the observed frequency of occurrence obtained from study of 200 offspring from breeding pairs with the same genotype as shown below (male $DRA^{-/-}$, $DRB^{-/-}$, $COLIVA3^{-/-}$ and female $DRA^{Tg/-}$, $DRB^{Tg/-}$, $COLIVA3^{+/-}$). These parent genotypes were chosen for optimal breeding of the female $DRA^{Tg/-}$, $DRB^{Tg/-}$, $COLIVA3^{-/-}$ genotype, which was required for experimental purposes. Highlighted in red are the genotypes skewed towards female mice, while in blue is the genotype skewed towards male mice.

	Male								Female							
	A -/-								A +/-							
	B -/-								B +/-							
	$\alpha 3$ -/-								$\alpha 3$ +/-							
Possible genotypes from breeding pair																
Male	Male	Male	Male	Male	Male	Male	Male	Female	Female	Female	Female	Female	Female	Female	Female	
A +/-	A +/-	A -/-	A -/-	A +/-	A +/-	A -/-	A -/-	A +/-	A +/-	A -/-	A -/-	A +/-	A +/-	A -/-	A -/-	
B +/-	B -/-	B +/-	B -/-	B +/-	B -/-	B +/-	B -/-	B +/-	B -/-	B +/-	B -/-	B +/-	B -/-	B +/-	B -/-	
$\alpha 3$ +/-	$\alpha 3$ +/-	$\alpha 3$ +/-	$\alpha 3$ +/-	$\alpha 3$ -/-	$\alpha 3$ -/-	$\alpha 3$ -/-	$\alpha 3$ -/-	$\alpha 3$ +/-	$\alpha 3$ +/-	$\alpha 3$ +/-	$\alpha 3$ +/-	$\alpha 3$ -/-	$\alpha 3$ -/-	$\alpha 3$ -/-	$\alpha 3$ -/-	
Expected Frequency of Occurrence																
6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	
Observed Frequency of Occurrence																
0%	1%	4.5%	11%	0%	0%	0.5%	8%	32.5%	2%	11%	16%	6.5%	0.5%	2%	4.5%	

4.2.4. – Flow cytometry analysis of MHC/HLA expression by lymphocytes from transgenic mice

Flow cytometry was used to investigate the cell surface expression of MHC/HLA DR15 molecules on lymphocytes from transgenic mice. Lymphocytes from two transgenic DR15 A3v mice (IAB negative, HLA DRAB positive) were compared with lymphocytes from two normal C57BL/6 mice (IAB positive, HLA DRAB negative), each stained and measured in triplicate. The results are shown in figure 4.2.8. and confirm the phenotype expected for PCR-determined genotypes for each mouse. 8.6% and 4.74% of cells isolated from lymph nodes of C57BL/6 mice expressed the mouse class II molecule (figure 4.2.8B-C), while 1.63% and 0.75% expressed the HLA-DR15 molecule (figure 4.2.8D-E). This is in direct contrast to the lymphocytes isolated from the lymph nodes of DR15 A3v mice from which 15.52% and 16.49% of the cells isolated expressed the HLA DRAB molecule (figure 4.2.8I-J), while 1.14% and 0.87% only appeared to express the mouse class II IAB molecule (figure 4.2.8G-H). All of the figures have the positive staining overlaid on cells stained with the relevant isotype control to allow for antibody-type non-specific binding. These data showed that while the DR15 A3v mice did appear to be MHC class II positive, there was also some reactivity with the mouse class II antibody. This suggests that either the gene has not been knocked out properly, although this is unlikely as the PCR reactions were WT negative for these animals, or that there is some cross-reactivity between the anti-mouse class II antibody and the MHC class II molecules on the DR15 A3v mouse lymphocytes.

Figure 4.2.8. – FACS plots of DR15 A3v lymphocytes compared with those from a C57/Bl6 mouse stained for HLA-DR and mouse class II.

This figure shows forward scatter and side scatter plots of unstained lymph node cells (figures A and F), plus overlay histogram plots of a population of cells bearing mouse class II (figures B and C, G and H) or HLA-DR15 (figures D and E, I and J) determined by fluorescence detected on FL1 for lymph node cells from a C57BL/6 mouse (figures A-E) and a DR15 A3v mouse PCR genotyped as mouse class II KO and HLA DRAB positive (figures F-J). Filled histograms show stained isotype control cells, while unfilled histograms show stained cells.

Figure A

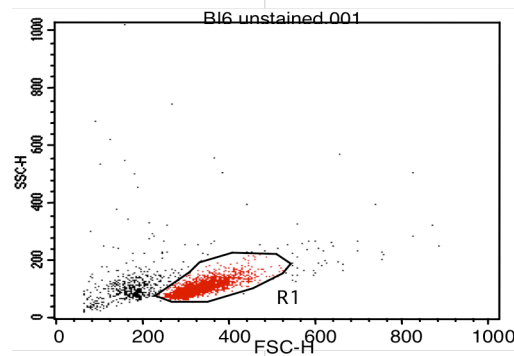


Figure B

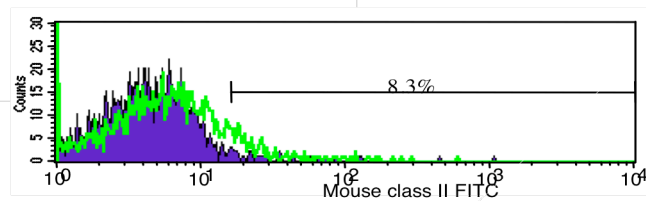


Figure C

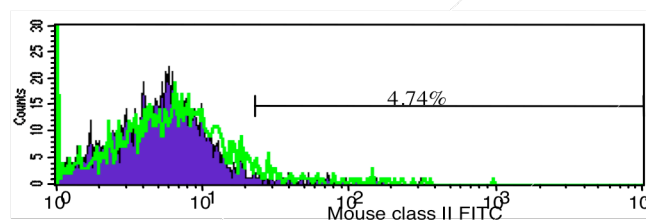


Figure D

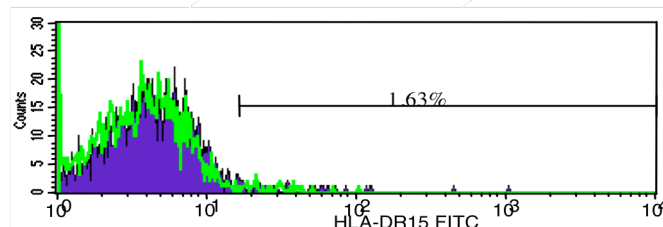


Figure E

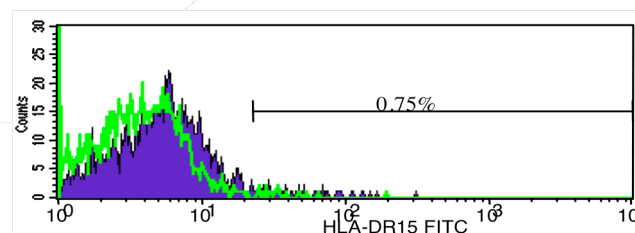


Figure 4.2.8. – FACS plots of DR15 A3v lymphocytes compared with those from a C57/Bl6 mouse stained for HLA-DR and mouse class II.

Figure F

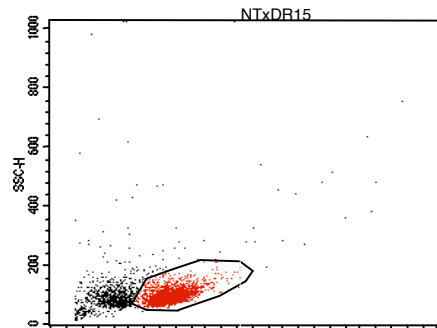


Figure G

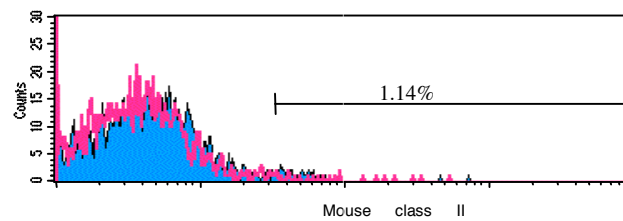


Figure H

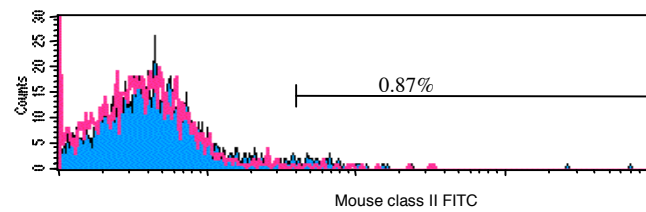


Figure I

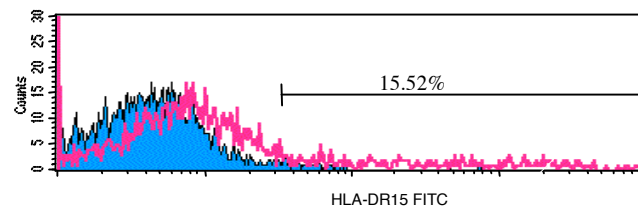
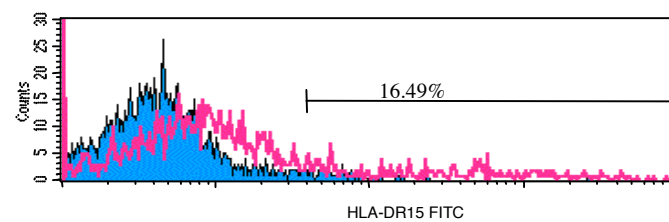


Figure J



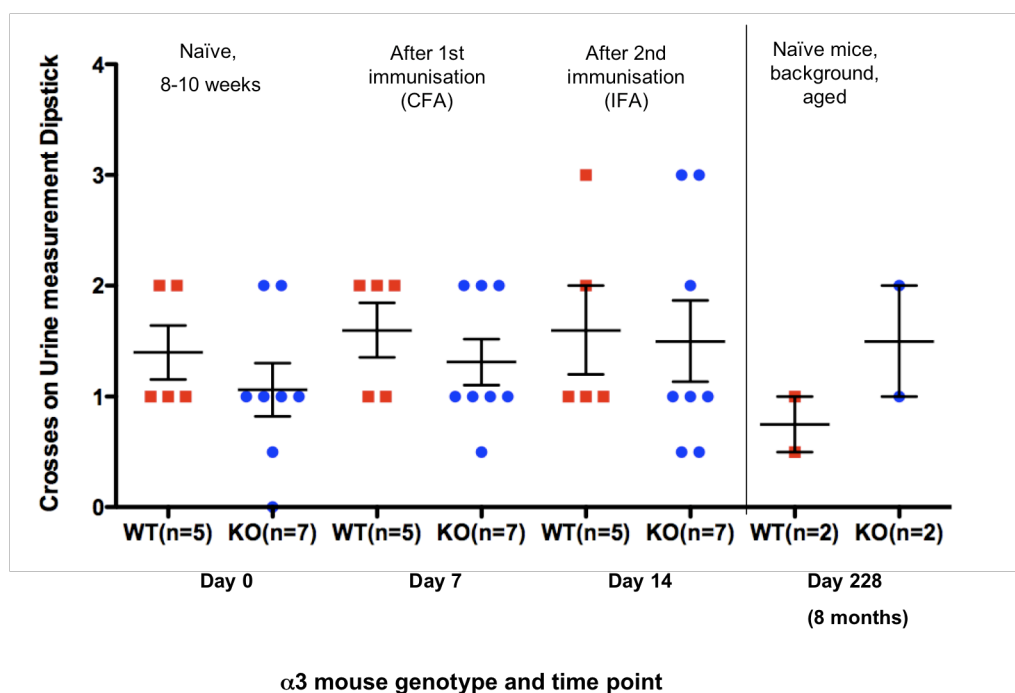
These cell suspensions were also fairly crude preparations containing all cells disaggregated from whole lymph nodes, so the relatively high background staining and numbers of unstained cells may be due to other, contaminating cell types, such as B cells and monocytes.

4.2.5. – Renal function of DR15 A3v transgenic mice

It has been noted that, if allowed to die from natural causes, $\alpha 3$ KO mice die younger than their $\alpha 3$ Het or $\alpha 3$ WT littermates. However, it is not known whether this is due to renal failure, or some other reason. Since the DR15 A3v mouse line was bred to be more susceptible to glomerulonephritis and renal failure (through the DR15 link to Goodpasture's disease and the $\alpha 3(\text{IV})$ KO likeness to Alport's disease), it was decided to examine whether renal function was impaired pre- and post-immunisation with antigen, and how this differed between $\alpha 3$ KO mice and their $\alpha 3$ WT littermates. Three different time points (before immunisation, after one immunisation with complete Freund's adjuvant and after the second "booster" immunisation with incomplete Freund's adjuvant) were used for each mouse, and renal function was estimated by determining the protein and blood content of the urine sample using Bayer urine dipsticks. There was a clear increase in proteinuria and haematuria for both $\alpha 3$ KO and $\alpha 3$ WT mice, but it was not determined whether this was due to the Freund's adjuvant used in the immunisation schedule. There was a more rapid escalation of proteinuria observed in $\alpha 3$ KO mice compared to $\alpha 3$ WT mice, although none of the differences were found to be statistically significant, either between WT and KO at each time point or between time points within each

Figure 4.2.9. – Scatter plot showing the extent of proteinuria in $\alpha 3$ WT and $\alpha 3$ KO mice at different time points following immunisation against background.

This scatter plot shows the different readings of proteinuria from different mice before, during, and after immunisation with $\alpha 3$ in adjuvant and compares $\alpha 3$ WT with $\alpha 3$ KO mice (mean and standard error marked). In addition, to determine how the immunisations had an effect on proteinuria in these animals, urine samples were taken from two naïve animals of each genotype at 8 months of age to determine the level of proteinuria in aged animals and whether it increases with age.



genotype ($P=0.3-0.9$). The immunised $\alpha 3$ WT mice appeared to have increased proteinuria after immunisation with antigen in complete Freund's. This increase in proteinuria was followed by reduction (although not back down to baseline levels) after the second immunisation using incomplete Freund's as the adjuvant. Additionally, the measurements were not quantitative as they were collected using urine dipsticks designed for human levels of proteinuria and haematuria, with larger volumes of urine, and were measured by eye against a colour chart. The data obtained is shown in figure 4.2.9, with a calculated mean and standard error of the mean shown. The differences seen in these samples are not statistically significant, either when comparing wild-type to knock out at each time point ($P=0.348$, 0.3961 and 0.8577 at day 0, 7 and 14 respectively) or each time point to baseline (WT $P=0.5796$ and 0.6847 for day 7 and day 14, KO $P=0.4458$ and 0.337 for day 7 and 14), by unpaired T-test. The sample size shown here is also very small without control, unimmunised mice at the same time points, so this work needs further investigation before a real conclusion can be drawn.

Since these mice were difficult to breed and obtain numbers sufficient for conclusive proof, this data should be treated as pilot data and more experiments should be done to determine the degree of impairment of renal function by means of increased creatinine in urine samples from these $\alpha 3$ KO mice.

4.3. – Discussion

The DR15 A3v line has been bred such that it does not express mouse class II molecules, can express the HLA-DR15 molecule - the HLA molecule known to be

expressed by 80% of Goodpasture patients - and can either express $\alpha3(IV)NC1$ collagen or be knocked out for the same molecule. While this mouse line does not develop Goodpasture's disease (at least with the conditions to induce immune responses used throughout this thesis), it should enable some elaboration on the immune responses generated by the Goodpasture antigen. This line is therefore a valuable resource for exploring specific immune features of Goodpasture's disease, most particularly antigen presentation by the HLA-DR15 molecule, and to determine how tolerance influences peptide presentation and immune response.

It is worth noting that difficulties in breeding this line are likely to be constant – care must be taken to ensure the correct genotypes are used for breeding pairs or there is a risk of losing either the MHC class II transgene from the line, or the $\alpha3(IV)NC1$ genotype. In particular, no male mice (0/200) have been identified to carry the HLA DRAB double transgene or the HLA DRB gene – it seems likely that this is due to the genotype being either incompatible with the male phenotype or being embryonic lethal for males. The former reason could include insertion of the HLA DRAB genes into a gene such that expression is incompatible with becoming male and forcing a more female appearance despite the XY genotype. An extreme example of this could be that the HLA-DRB gene could have been inserted into the testosterone gene, disrupting testosterone production and resulting in offspring female in appearance. This does not appear to be the case in the DR15 A3v line as these “females” would not be suitable for breeding or experimentation and would have different physiology from the true females, neither scenario was observed. Females with the correct genotype by PCR for the HLA DRAB are capable of breeding, whereas if they were genetically male, this would not be the case. In

reality, the limiting factor for breeding was the $\alpha 3$ status of the female, as described above. Without more specific data regarding the location of the insertion (for example, fluorescent in-situ hybridisation (FISH)), only conjecture is possible, however it is known that the insertions are not linked to the sex chromosomes. If the insertion was only in the Y-chromosome, all male mice would have the gene and this is known to be untrue, as the mice are negative by PCR for the gene. If the insertion was into the X-chromosome, 50% females (rising with time and subsequent in-breeding) would have the insertion and some males would also have the insertion, which is also known to be untrue.

As discussed earlier, additional reasons for lack of males with the DRAB genotype could also be due to segregation to chromosomes, however, if this were random chance of segregation during gametogenesis, it would be expected that some males would be born that express the double HLA DRAB transgene. In the 200 mice counted here (figure 4.2.7.), males expressing HLA DRAB are never born, and in a total of more than 750 mice (all mice born, irrespective of $\alpha 3$ status of parent mice) this is also true (0/750). This therefore further supports the suggestion that the HLA DRAB double transgene is incompatible with male mice.

More work to study where the recombination event occurred on the genome following insertion of the HLA DRAB transgenes would help to understand the mechanisms and genes affected by the insertion events. Genes studied that cause embryonic lethality in males include *sxl* in *Drosophila melanogaster* – *Drosophila simulans* hybrids (218) and sex-specific differences in embryonic lethality have also been observed in a mouse line containing a variegating green fluorescent protein (GFP) transgene. Females with homozygous knockout died mid-gestation and were

found to display abnormal methylation on the X chromosome, resulting in aberrant gene activity (219).

Generally speaking, X-linked recessive disorders are more likely to be carried by females and be apparent in males (for example colour-blindness and Angelman syndrome), but it has been more difficult to pinpoint genes resulting in male-specific lethality. One gene known to show such characteristics is the human *IKK γ* gene located at Xq28 – defects in this gene cause Incontinentia Pigmenti (also known as Bloch-Sulzberger Syndrome), a disorder that causes prenatal death in males and variable skin pigmentation combined with retardation and tetraplegia in females. The affected females show highly skewed X inactivation, with variable degree of corresponding disease (OMIM number 308300) (220). It is not likely that this is the gene affected by insertion into the DR15 A3v mouse genome, as the female mice do not show any such abnormality, but defects in this gene do illustrate the potential for male-specific embryonic lethality.

In the DR15 A3v line, females are therefore the sole source of the HLA DRAB double transgene and must also be COLIVA3 heterozygotes as COLIVA3 KO females die during pregnancy or immediately post-natal. The breeding genotype is the most commonly observed and was seen to occur 32.5% of the time. While the COLIVA3 gene is of interest in terms of exploring specific immune responses to the protein encoded by that gene, the development of the mouse line in which the CD4 T cell repertoire is restricted to the HLA-DR15 molecule is also of interest in exploring immune responses seen during other autoimmune diseases, in particular multiple sclerosis (175).

While this mouse line appears to be an appropriate system to investigate the immune responses to the $\alpha 3(\text{IV})\text{NC1}$ protein, there are additional factors to bear in mind when designing experiments. In particular, the fact that the mouse $\alpha 3(\text{IV})\text{NC1}$ has only 92% homology with the human and while the mice homozygous knock-out for the COLIVA3 gene may develop similar immune responses to the human $\alpha 3(\text{IV})\text{NC1}$ protein, it is possible that mice that still express the COLIVA3 gene show different immune responses to the human $\alpha 3(\text{IV})\text{NC1}$ protein compared with their self-protein. It is also possible that, irrespective of tolerance, an aberrant immune response may occur solely due to slight differences in the COLIVA3 gene sequences of mice versus human.

This line also has other limitations in that there is variability within the line. The COLIVA3 KO line was originally on the 129sv background, while the DR15 line was on a C57BL/6 x CBA background. While both of these backgrounds are inbred over hundreds of generations, by cross-breeding the two any advantage of inbreeding has been removed, so genetic variability becomes more apparent. In the DR15 A3v line, variation was observed by coat colour (ranging from grey, agouti to black). This variation in coat colour was removed following rederivation of the line when moving the animals to a new, clean facility, and selectively choosing agouti (the most prevalent coat colour) as the breeding pairs and source animals for the rederivation project. As a general rule, it takes 16 generations for genetic variation between parents and offspring to be reduced to negligible (221). That being the case, if each generation is counted from when the previous generation dies, and assuming that each generation will live 6 months, it could take 8 years to reduce genetic variation to nothing in this line. If counted from when each generation breeds, and

provided each generation only breeds within that generation, this time period could be reduced to 4-5 years, but on the proviso that no back-crossing with previous generations was undertaken.

Other rodent lines do exist to specifically investigate Goodpasture's disease – in particular, work undertaken using rats enabled the autoimmune aspects of the disease by transfer of autoimmune serum to naïve animals and resultant disease in those animals (222, 223) and enabled the identification of specific autoantibodies (224). These lines have also been used to explore the cryptic properties of the Goodpasture antigen (168, 223) and to therefore identify regions of immunological interest. It has been possible to induce glomerulonephritis in some of these models (174, 224), but this required a longer time period of *in vivo* incubation prior to culling the animal and determination of glomeronephritis by histopathology.

To summarise, a murine transgenic line with restricted class II repertoire (human HLA-DR15 molecule) and with or without $\alpha 3(\text{IV})\text{NC1}$ protein expression was developed. This transgenic murine DR15 A3v line is used to determine specific immune responses to a specific antigen and two specific genotypes were used: mouse class II KO, HLA-DRA and -DRB positive, $\alpha 3$ WT and mouse class II KO. HLA-DRA and -DRB positive, $\alpha 3$ KO, in order to compare immune responses both in the presence and absence of tolerance. This transgenic line could help elucidate mechanisms that are prevalent in the development of Goodpasture's disease. It is also possible that similar mechanisms may cause other autoimmune diseases with a known HLA-restriction, so this transgenic mouse line could indirectly assist in determining possible causes and targets for therapy in other, more prevalent autoimmune conditions.

Chapter 5 – Antigen Preparation

Chapter 5.1. - Introduction

The main aim of my project was to determine immune responses to a specific antigen in the presence and absence of tolerance. To do this, transgenic mouse lines, with and without tolerance, were immunised with Goodpasture antigen, the non-collagenous domain of the $\alpha 3$ chain of type IV collagen ($\alpha 3(\text{IV})\text{NC1}$) and the immune responses against overlapping peptide fragments of this protein were tested. In order to do this, sufficient quantities of qualitatively adequate protein were purified from recombinant bacteria or purified from human tissue.

Numerous methods exist for producing recombinant protein, including plants (225), mammalian cells or insect cells *in vitro* (226), or using bacterial expression systems. In addition, it is possible to extract the $\alpha 3(\text{IV})\text{NC1}$ protein from native tissue in this case, kidney tissue. This method of extracting protein does not produce recombinant proteins and produces the $\alpha 3(\text{IV})\text{NC1}$ reasonably close to the native conformation. All of the methods have advantages and disadvantages and these were considered before deciding which technique was most appropriate for this thesis.

Producing this particular protein in a plant system has not been attempted as this technique would produce insufficient quality and quantity of the $\alpha 3(\text{IV})\text{NC1}$ and would require considerable initial outlay making this technique uneconomical (225). This protein has been produced in a transfected insect cell system, both within my laboratory, and by other groups (227), but was relatively inefficient. Although this

method could produce sufficient protein for the experiments planned, it was decided that two other well-established methods would be more appropriate – extraction from tissue and use of transformed bacteria. It was also decided to bring another well-established method to this research group by developing an $\alpha 3(\text{IV})\text{NC1}$ -containing construct for transfecting the human embryonic kidney cell line. Each of the chosen techniques has advantages and disadvantages which are discussed here in more detail.

5.1.1. – Production of recombinant protein by bacteria

A general method for enhanced production of recombinant protein by bacteria was first described in 1979 (228) and has since been used for mass production of protein. This paper described a method to insert the lac Z promoter at any distance upstream of a cloned gene and produce large amounts of protein. In this case, the cro protein (cro being expressed by the bacteriophage λ and inhibiting expression of some genes within the infected bacteria) was tested and this technique achieved up to 190, 000 cro protein molecules per cell.

There are additional factors to consider before making large amounts of recombinant protein. These include assessing inconsistency within and between batches of proteins manufactured in this way. For example, proteins produced in bacterial recombinant systems have a different conformation, both from the native protein, and from each preparation, which can yield multiple conformations. This can also be the case when using the same transformed bacterial strain, under identical growth conditions, and the same glycerol stock. This is due to the fundamental differences between bacterial and mammalian cell protein production mechanisms.

One of these major differences is that bacteria use different protein-folding chaperones with structural preferences that differ from mammalian protein-folding chaperones. Another major difference is that bacteria do not possess the capability to form disulphide bonds between cysteine residues thus producing proteins with differing conformations. The cysteine residues form disulphide bonds with other available cysteine residues irrespective how the native protein folds (229). This also means that proteins with very specific conformational dependence are not suitable for production using this technique. As an example, the $\alpha 3(\text{IV})\text{NC1}$ contains 12 cysteine residues, so while it is possible to produce this protein in a recombinant system, there is variation both within and between batches. In addition, the recombinant bacterial proteins are not glycosylated as bacteria (with the exception of *Campylobacter jejuni*) do not have the cellular machinery to glycosylate the N-terminal end of proteins (230). All work undertaken in this thesis using recombinant $\alpha 3(\text{IV})\text{NC1}$, has utilised protein produced within the same batch.

Despite the differences in structure and protein conformation seen when using a bacterial production system, the use of bacteria to produce protein does have advantages. In particular, bacterial cultures are easy to grow and maintain and there are multiple vectors available that can be inserted into the bacteria through a process called transformation. These vectors can be manipulated to contain the gene which encodes for the protein of interest, and, depending on the vector used, the protein production (as in the construct used for transforming this bacterial stock) can be induced at specific time points. This enables the production of large quantities of the protein in short periods of time and with relatively little cost. The protein can also be

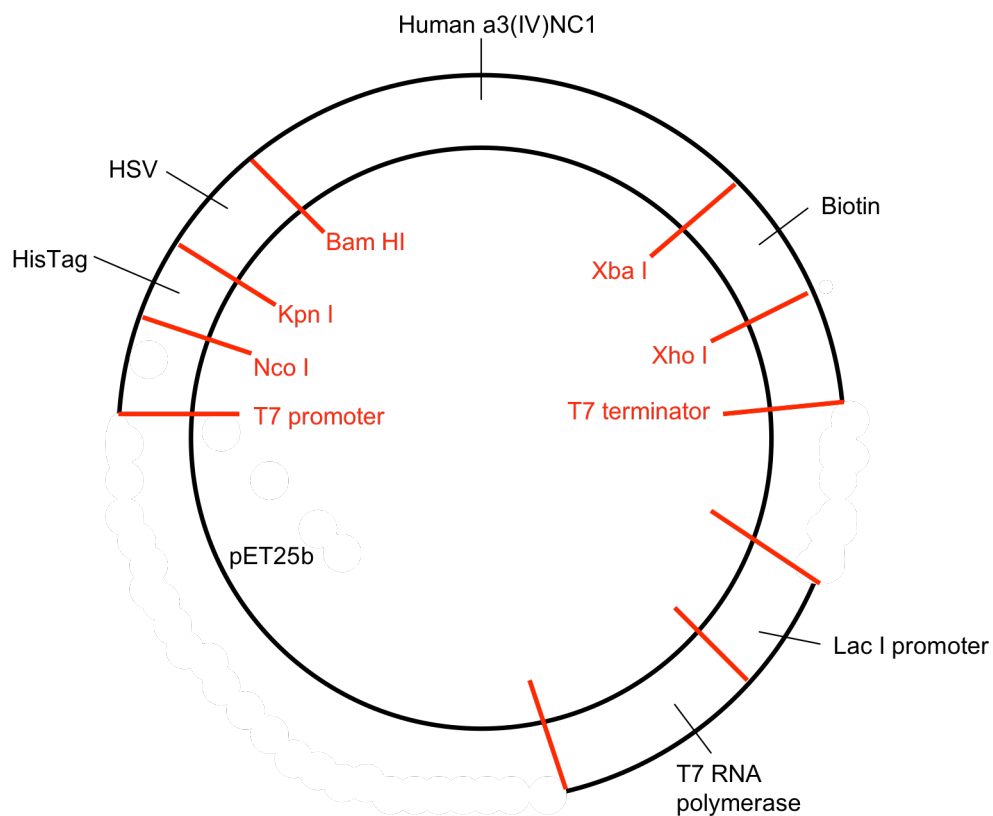
‘tagged’ to target its location to a specific region of the bacterium post-production, for example into the periplasm or into inclusion bodies.

The bacterial strain BL-21 was used for $\alpha 3(\text{IV})\text{NC1}$ production, a strain of *E. coli* particularly suited to expression of large amounts of foreign protein after successful transformation. BL-21 bacteria do not express the *Ion* or the *ompT* proteases ensuring that the protein produced remains intact. BL-21 *E. coli* also contains a mutant gene for lac permease (*lacY*) that ensures uniform entry of inducing agent (IPTG), thus producing an IPTG concentration-dependent homologous level of protein expression. In this project, BL-21 bacteria were transformed using the plasmid pET25b containing a construct designed to direct expression of $\alpha 3(\text{IV})\text{NC1}$ with a histidine tag, an HSV tag and a biotinylation tag (schematic of construct, Figure 5.1.1.). This plasmid also contains various restriction sites that can be exploited to add extra components to the protein, should they be required (for example a fluorescent tag such as green or yellow fluorescent protein – GFP or YFP).

While it is not the most common use for the technique, bacterial protein expression systems have been used for the production of protein for use in medical applications. The most well known example of this is the production of insulin for the treatment of type I diabetes. Other proteins produced in this way include rennin (or prochymosin, for use in culturing cheese) and production of the alkaline proteases used in dehairing hide to make leather. Bacterial protein expression systems can also be used for production of enzymes and proteins for use in the food, leather and pharmaceutical industries (231-233).

Figure 5.1.1. – Schematic of the plasmid used to transform BL21 *E. coli* to produce recombinant $\alpha 3(IV)NC1$.

This diagram illustrates a schematic of the plasmid used to transform the BL21 strain of *E. coli* bacteria. Induction with IPTG drives production of $\alpha 3(IV)NC1$ within inclusion bodies. The plasmid not only contains the human $\alpha 3(IV)NC1$ sequence, but also has a histidine tag, an HSV tag, and a biotin sequence within the expression vector, pET25b. The vector pET25b uses the highly specific T7 promoter system for production of protein and contains a second sequence under the control of *lacI* to produce the T7 RNA polymerase.



This application of the technology to produce protein emphasises the advantage of producing large quantities of protein from a relatively small preparation. However the extraction procedure is different to that used for extracting native antigen so the resultant protein preparations are not strictly comparable.

5.1.2. - Extraction of antigen from tissue

Extraction of antigen from human tissues has the distinct advantage that the protein conformation is in a similar conformation to the same protein found in the body. However, there are also major disadvantages in terms of availability of tissues for protein extraction, and the yield of the protein extracted is usually small. If the protein can be extracted from blood there is usually greater accessibility, but there is still a reliance on either volunteer donors or patients. In this project, the best source of human protein was the kidney. Although kidney samples were difficult to obtain, under certain circumstances it was possible to get human kidneys that had been rejected for use in transplant surgery by transplant surgeons (usually as result of damage to the blood vessels entering or exiting the organ (168, 179)), but offered for research by the donor. Whilst not an ideal source of material, this was the only option available during the course of this project for native human antigen extraction.

Another significant disadvantage in this system is the amount of time required to purify the tissue and to produce significant amounts of native protein. As a result, it is a very expensive process. The extraction process, as described in Chapter 2, is time consuming with manual disaggregation, sonication and enzymatic digestion steps. None of these processes are known for being gentle, even if the destruction of

tissue is effective enough to extract the requisite protein by chromatographic means. In brief, the cortex was stripped off the whole kidney and sieved through specific sized filters to isolate whole glomeruli (179). These were then sonicated to destroy the glomerular structure and frozen prior to enzymatic digestion of the suspension. The freezing also helped destroy the glomerular structure, while the collagenase treatment released the soluble non-collagenous domains.

Column chromatography is a well-established technique for separating out proteins according to specific characteristics, be it size, charge, binding capability or binding to protein-specific immunoglobulins (224, 234). In ion-exchange chromatography, the conditions can be manipulated such that the protein of interest remains bound to the column until it has neutral charge and is released from the column and collected. In purifying the $\alpha 3(\text{IV})\text{NC1}$ protein from human kidneys, two specific characteristics of the protein were exploited: the overall insolubility of the protein; and the high positive charge on the protein. In order to elute this protein, a high salt concentration (1M) and high pH (pH 10), in concert, were required.

These procedures, while allowing me to isolate some of the protein required for my project, may also have destroyed the protein structure by mechanical, sonic or enzymatic means prior to manipulation and purification using solutions in which it should retain its native conformation and properties. However, if the structure had been completely destroyed, it would also follow that the properties associated with the protein in its native state would also be lost, so care must be taken when eluting the extracted protein, and the different fractions should be tested, by Western blot for ability to bind Goodpasture patient sera.

5.1.3. – *Production of recombinant protein by eukaryotic cells*

Production of recombinant protein by transfected mammalian cells reduces the problem of producing protein in an altered conformation, but may still result in a different glycosylation state and, for the $\alpha 3(\text{IV})\text{NC1}$ protein, may produce issues with variable trimerisation since the most energy efficient state for the $\alpha 3(\text{IV})\text{NC1}$ protein is as part of a trimeric molecule. This can be a major drawback to producing the protein satisfactorily *in vitro*. The $\alpha 3(\text{IV})\text{NC1}$ protein naturally interacts with other α chain proteins to maintain specific structural features. As a result, when expressed in isolation by transfected mammalian cells, the $\alpha 3(\text{IV})\text{NC1}$ protein may well have a different conformation compared to native proteins in conjunction with the relevant associated proteins. Producing the $\alpha 3(\text{IV})\text{NC1}$ domain alone will almost certainly result in some conformational differences, even if spontaneous polymerisation does not occur when the protein is secreted.

The technique for producing proteins from overexpressing mammalian cells was first described in 1982 by Schumperli *et al.* (235) and was developed in order to produce and purify more galactokinase within mammalian cell systems. This was found to be a transient transfection (i.e. the protein is only expressed temporarily over a short period of time), but could be a stable transfection if an additional dominant genetic marker was cotransfected (for example, a gene for antibiotic resistance and the cells grown in the presence of that antibiotic). Under these conditions the transfected mammalian cells have the potential to produce more protein than would be derived from native tissue, but in a similar conformation to native protein. This technique neatly bridges bacterial recombinant expression systems and protein extraction from native tissue but also has drawbacks, in

particular the time involved in selecting the correctly transfected colony that produces sufficient protein. This process involves the growth of the cells in selective media and testing the media (in this instance) for the secreted protein (as described in Chapter 2). The most accurate way to do this is by Western blotting, using non-denaturing gels, and probing the transferred proteins for the protein in question, but with fairly large numbers of supernatants to test, this can be relatively time-consuming. In addition, antibodies are very conformation-dependent, so to determine existence of correctly folded protein, a very conformation specific antibody should be used to screen the supernatants obtained from the transfected colonies. There is another issue with detecting protein by Western blotting – some proteins change conformation when transferred to nitrocellulose. In that situation, it has been shown that altering components of the transfer buffer or renaturing gels after electrophoresis can help reduce the detrimental effects on the protein (236). It is also best to test supernatants without serum so small amounts of secreted protein can be detected.

Since the $\alpha 3(\text{IV})\text{NC1}$ protein retains the ability to bind patient sera on Western blots, this also enables rough quantitation of the amount of protein produced, provided a concentration curve of known amounts of protein is also probed on the same blot. The protein concentration was also tested on a spectrophotometer to verify the concentration determined from the Western blot.

As mentioned above, transfected cells can either show transient expression of protein, or form stable transfects and grow into a colony of successfully transfected cells that permanently express the protein. As is also mentioned above, this depends on the other genes or proteins that are co-transfected with the gene of interest. For

stable transfects, the plasmid construct usually also contains a gene for some method of selection, for example antibiotic resistance. This enables selection of successfully transfected cells/ colonies and also ensures stable transfection of the plasmid construct as cells that express the antibiotic resistance genes have a survival advantage.

Chapter 5.2. – Results

5.2.1. – Production of recombinant $\alpha 3$ from bacteria

Production of the recombinant protein involved growth of transformed BL21 *E. coli* in a 2 litre culture, followed by extraction of the protein from the bacteria. This is described in full in Chapter 2, but in brief, involves growth of transformed bacteria to an optical density (OD₅₅₀) of 0.6, and induction of protein expression with IPTG. The bacteria were then pelleted and subjected to enzymatic digestion (using hen egg lysozyme, DNase, magnesium chloride and deoxycholic acid) in order to isolate the inclusion bodies and digest other cellular proteins. Inclusion bodies are very robust so were then sonicated in the presence of detergent (Triton-X100) in order to lyse the bodies and release the induced $\alpha 3(\text{IV})\text{NC1}$ protein. This was then purified by ion-exchange chromatography and the fractions from each stage of the chromatography process were run on a 12% acrylamide gel prior to staining, to test for the presence of the $\alpha 3(\text{IV})\text{NC1}$ protein. This process is illustrated in Figure 5.2.1A. The second gel shown in this Figure shows serial dilutions of successfully

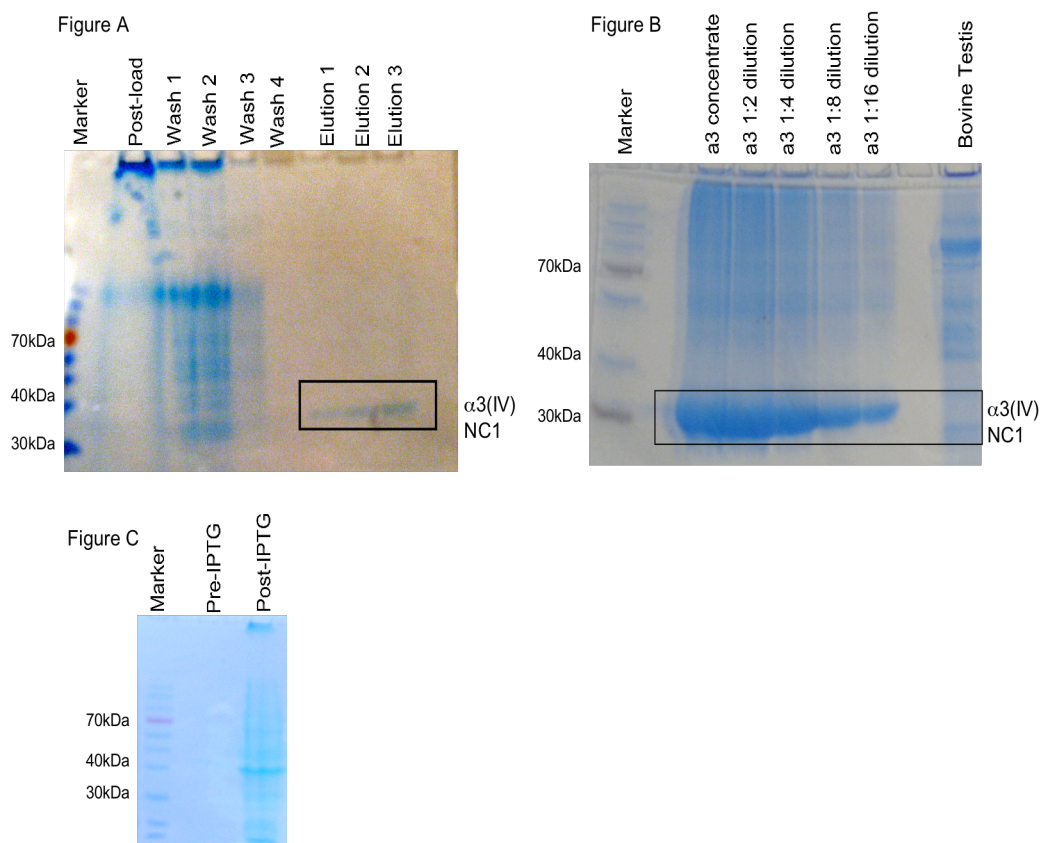
purified recombinant $\alpha 3(\text{IV})\text{NC1}$ and compares it to the positive control of bovine testis. As can be seen from the gel, this preparation is far more concentrated and was used as the recombinant $\alpha 3(\text{IV})\text{NC1}$ in all subsequent work. The total yield was 130mg $\alpha 3(\text{IV})\text{NC1}$ from 2 litres of bacterial culture.

5.2.2. – Isolation of native $\alpha 3$ from tissue

The recombinant protein preparation from bacteria which was used in subsequent experiments produced approximately 65mg/l LB broth recombinant human $\alpha 3(\text{IV})\text{NC1}$, whereas $\alpha 3(\text{IV})\text{NC1}$ extracted from the cortex of 5 human kidneys, not an insubstantial amount of tissue, resulted in a yield of only 1.2mg $\alpha 3(\text{IV})\text{NC1}$ (for Western blots and chromatograms see Figures 5.2.2 and 5.2.3. respectively). The total amount of collagen IV was not measured as this would have required lyophilising the solubilised NC1 domains and potentially damaging the protein in the process. In addition, the protein is relatively insoluble. Even without lyophilisation, the protein is only stable in 8M urea or 6M guanidine hydrochloride. Since the human $\alpha 3(\text{IV})\text{NC1}$ protein should be soluble, this problem with getting the protein into suspension suggests different conformation and tertiary structure rendering the protein highly hydrophobic.

Figure 5.2.1. – Coomassie-stained gels showing purified recombinant human $\alpha 3(IV)NC1$

Coomassie-stained gels showing $\alpha 3(IV)NC1$ purification using ion-exchange chromatography. Figure A shows aliquots removed at different stages of purification and shows (from left) the post-load, subsequent wash steps (1-4) illustrating the dirty nature of the preparation, and the $\alpha 3$ protein eluting within 3 elution volumes (within box). By comparison, Figure B shows a successfully purified, high yield preparation, in contrast with bovine testis (positive control). This is the preparation used in all subsequent experiments. Figure C illustrates the result of addition of IPTG to the bacterial colony used to express the $\alpha 3(IV)NC1$ protein.



Other ways to measure the total protein yield include weighing the whole contents and extrapolating the weight of the same volume of suspending buffer (a very classical method), or by measuring the actual protein concentration using commercially available kits that use spectrophotometry to measure a proportional colorimetric reaction to protein concentration. Both of these methods are potentially useful, however, weighing the sample would not specifically measure the protein in solution, while the BCA assay would not give an accurate result due to the amount of urea also within the solution.

The disparity in amounts of protein obtained can be partially explained by the fact that the $\alpha 3(\text{IV})\text{NC1}$ chain is one of the more scarce collagen IV chains within the kidney, and a more accessible source of $\alpha 3(\text{IV})\text{NC1}$ was very much required. The low yield also suggested loss of protein at a stage during the purification process. This is most likely to be during the enzymatic digestion and wash stages prior to ion-exchange chromatographic purification. These stages are the least regulated and defined, but in order to check this theory, tests would need to be run on samples from every stage of the process, and normalised for protein content.

Figure 5.2.2. – Stages in the purification of human $\alpha 3$ NC1 domains from kidneys.

Figure A shows glomeruli isolated from human kidneys prior to extensive sonication, Figure B shows the resultant solution post-sonication (microscopic analysis, x400 magnification). Figure C shows the purified material by coomassie gel, a Western blot and on a Western blot and coomassie gel overlay, from left to right is prestained marker ladder, post-collagenase digestion (lane 1) and pre-collagenase digestion (lane 2). The $\alpha 3$ protein should show two bands, a ~ 28 kDa monomer and a ~ 50 kDa dimer, but always runs heavier on SDS-PAGE gels, probably because the isolated $\alpha 3(\text{IV})\text{NC1}$ protein is highly cationic and, once isolated, forms a different conformation to protect hydrophobic residues normally protected by the interactions with other α chains.

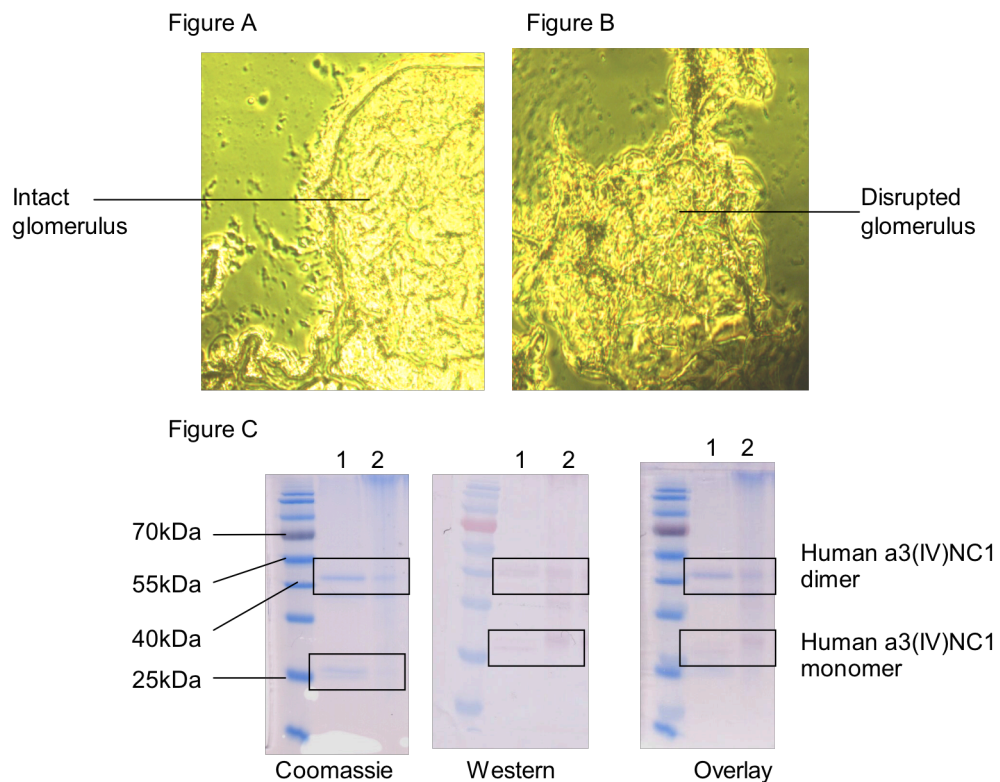


Figure 5.2.3. – Cation exchange chromatography profile for the isolation of the $\alpha 3(\text{IV})\text{NC1}$ from collagenase digests of human kidney.

This line chart shows the pH and salt gradients and the UV trace for protein as used in the isolation of the $\alpha 3(\text{IV})\text{NC1}$ protein. The salt gradient is plotted in blue, the pH gradient is plotted in green, the UV trace is pink, while fraction is plotted along the X axis. Most of the protein is eluted from the column during the initial wash stages and during the pH gradient, however the NC1 domain of the human $\alpha 3$ chain is highly cationic so is only eluted during a high pH and a high salt concentration, as shown below.

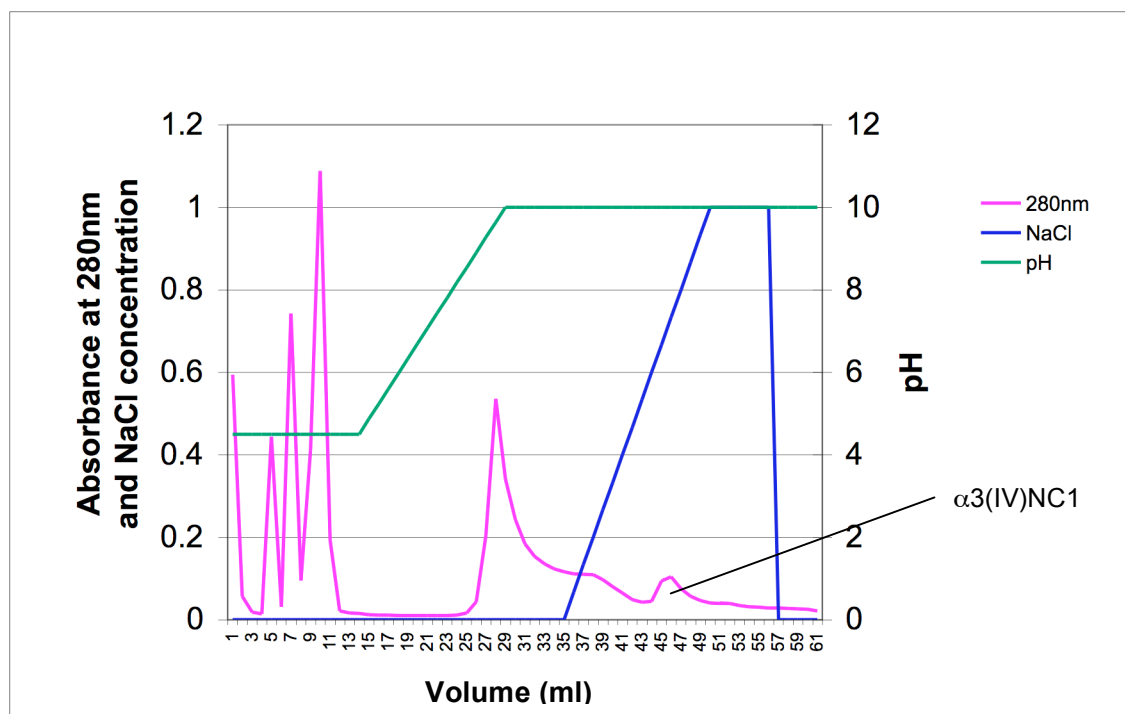


Figure 5.2.4. – Western blots of fractions following cation-exchange chromatography for the human $\alpha 3(IV)NC1$ domain.

These gels and blots compare unpurified NC1 domains with various protein containing fractions following cation-exchange chromatography. As can be seen in Figures A (coomassie) and B (Western blot), some fractions contained protein but tested negative for $\alpha 3(IV)NC1$, while Figures C (coomassie) and D (Western blot) show 2 fractions (24 and 25) positive for reasonably pure and much cleaner $\alpha 3(IV)NC1$ domains (inside boxes, equivalent to volumes 44-49ml on chromatogram in Figure 5.2.3.).

Figure A

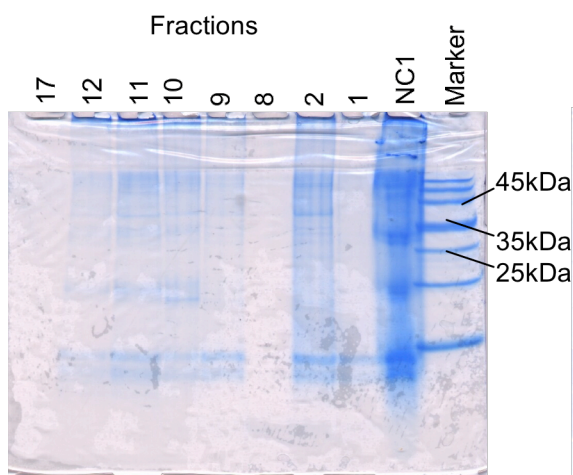


Figure B

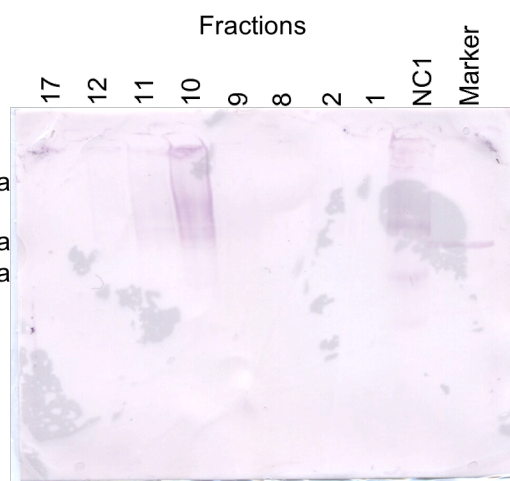


Figure C

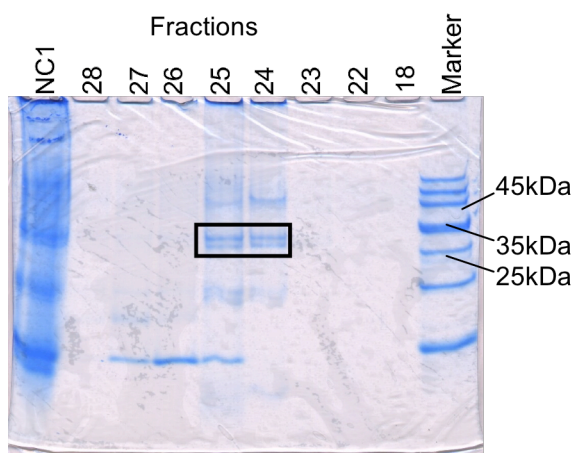
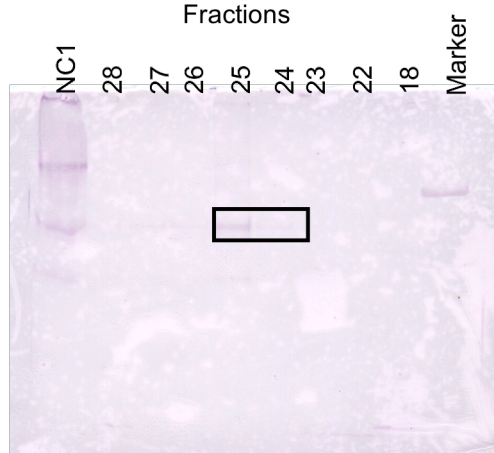


Figure D



5.2.3. – Production of $\alpha 3$ from mammalian cells

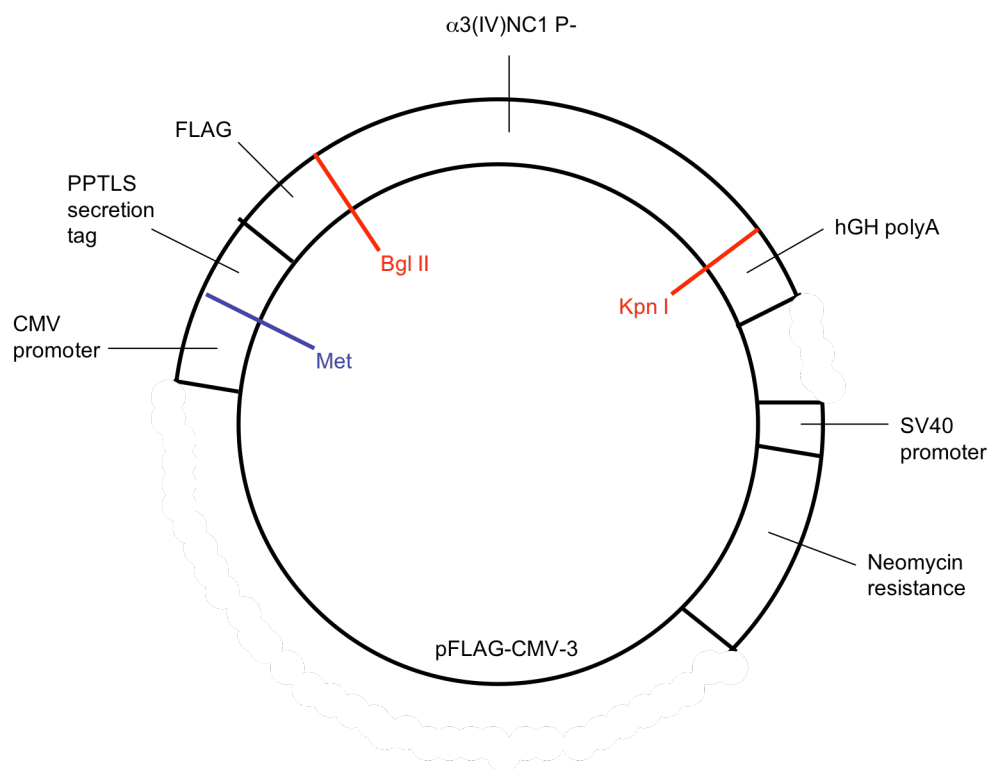
This knowledge and data emphasises the application of the third technique illustrated in the schematic in Figure 5.2.4. – transfection of mammalian cells. Although the yield of protein may not be as high as from a bacterial system, the overall conformation of the protein is more likely to be in the native state making any comparisons to the human disease processes, as in the case of this thesis, more relevant. It is, however more difficult to purify the protein and to maintain the cell line producing it effectively.

Figure 5.2.5. shows a schematic of the construct used in transfections to produce the Goodpasture antigen in human embryonic kidney cells. The vector used (pFLAG-CMV-3) is a commercially available vector with a secretion sequence on the N-terminal end of the protein (Sigma). Although the insert was more difficult to source, within the laboratory there were two constructs containing the sequence for $\alpha 3(\text{IV})\text{NC1}$ named P+ and P- respectively.

P+ is the $\alpha 3(\text{IV})\text{NC1}$ domain as found in patients with Goodpasture's disease as well as normal controls and is named for the presence of a PstI restriction enzyme site part way through the sequence. The P- construct lacks this PstI restriction site. I tried amplifying each construct by PCR, but was only able to amplify the P- insert successfully. This insert was then ligated into the pFLAG-CMV-3 vector in preparation for transfection into HEK293 cells (a human embryonic kidney cell line). The transfection process was performed 3 times but failed to produce a cell line capable of expressing the $\alpha 3(\text{IV})\text{NC1}$ protein in secreted form.

Figure 5.2.5. – Schematic of the construct used to transfect HEK293 cells and induce $\alpha 3$ production.

This schematic shows the pFLAG-CMV-3 plasmid with the $\alpha 3$ (IV)NC1 P- sequence insert. The plasmid contains a FLAG sequence (a marker tag), a gene for geneticin resistance (Neomycin) and a tag (PPTLS) that ensures extracellular secretion of the protein, when produced by a cell. The $\alpha 3$ gene is controlled by a CMV promoter, while the gene for neomycin resistance is controlled by an SV40 promoter. Shown in blue is the start methionine residue, while restriction sites from within the multiple cloning site and used to insert the $\alpha 3$ (IV)NC1 P- sequence are shown in red.



5.3. – Discussion

During the course of this project, particularly during the production of antigen for experimental purposes, there were many problems to overcome. In terms of the bacterial recombinant protein, there were problems with yield and there was definite inconsistency between batches of $\alpha 3(\text{IV})\text{NC1}$ produced by *E. coli*.

In obtaining human $\alpha 3(\text{IV})\text{NC1}$ domains from tissue, there were different obstacles to overcome – the major one being to minimise loss of protein throughout the extraction procedure. Losses become more significant when a dilute solution is used in multiple steps. In this procedure, the early steps involving sieving the tissue, sonication and enzymatic digestion of the sieved protein mixture produced a very viscous mixture that also increased the likelihood of loss at each step. As shown in the chromatogram (Figure 5.2.4.), there were very low levels of $\alpha 3(\text{IV})\text{NC1}$ present in the solution. This may be due to availability of the protein within the tissue; the $\alpha 3(\text{IV})$ is one of the more scarce type IV collagen chains, so less would be present in any human tissue-derived and collagenase-digested solution. Additionally, the losses described above would not just affect the $\alpha 3(\text{IV})\text{NC1}$ domains, but all the α chains isolated from the kidney tissue. This is most likely to be proportional, so the proportion of the $\alpha 3(\text{IV})\text{NC1}$ isolated, when compared to the other protein peaks on the chromatogram, is likely to be accurate. However, this was still a very low yield (1.2mg) compared to the amount of protein isolated in earlier peaks of the cation-exchange process (not measured, but illustrated in Figure 5.2.3.).

The technique used to purify the $\alpha 3(\text{IV})\text{NC1}$ protein from human tissue had not changed significantly from the method published by Spiro in 1967 whose group studied the chemical composition of the glomerular basement membrane (179). Since 1967, there have been several endorsements of this method (237-240) although there are associated difficulties with obtaining large amounts of human tissue. Newer techniques for larger scale production of the $\alpha 3(\text{IV})\text{NC1}$ protein including the production of recombinant protein by bacteria (241) and transfected cell lines that secrete the $\alpha 3(\text{IV})\text{NC1}$ protein into the surrounding media (166, 167) are quicker and less expensive in terms of reagents and starting material.

Problems with yield of the $\alpha 3(\text{IV})\text{NC1}$ protein from human tissue and high levels of variation between concentrations of recombinant protein batches produced by *E. coli* emphasised the need for a reliable and consistent source of the $\alpha 3(\text{IV})\text{NC1}$ protein for our group. In order to produce the protein, developing a transfected eukaryotic cell line that would secrete $\alpha 3(\text{IV})\text{NC1}$ in a comparable conformation to native protein in the native state was attempted. There were two possibilities for this: either transfection of an insect cell line to produce the $\alpha 3(\text{IV})\text{NC1}$ protein, or transfection of a mammalian cell line. The mammalian cell route was chosen, partly to utilise skills already available in mammalian cell culture, but also due to the number of papers published that used $\alpha 3(\text{IV})\text{NC1}$ and other $\alpha(\text{IV})$ chains produced in this way (166, 242, 243), plus the ready availability of reagents, suitable equipment and expertise within the research centre. While initial attempts at transfection were unsuccessful, the construct was produced in sufficient quantity and frozen so it would be possible for new attempts to be made in the future and could be a valuable resource for the group.

The purification techniques used during the antigen preparation mainly involved exploiting specific characteristics of the protein such that it would be the only protein isolated within a specific fraction. In particular, the high positive charge on the $\alpha 3(\text{IV})\text{NC1}$ protein enabled isolation by cation-exchange chromatography. While this does enable very specific isolation and purification, the volume in which the purified protein is eluted can be much greater than the original volume depending on the column volume. This lead to a need for concentrating the purified protein, either by ethanol precipitation, using concentrator spin columns (molecules above a certain molecular weight are retained by a filter) or by using dialysis tubing on dry polyethylene glycol, which then absorbs water from the protein solution. This is another area in which protein losses can occur, through inability to resuspend precipitated protein, the protein sticking to the filter or sides of the spin column or through the protein sticking to the dialysis tubing. In addition, the protein aggregates and gets dehydrated (particularly while using dialysis tubing), which will also encourage formation of protein complexes and encourage the protein to protect areas of importance, thus reducing the possibility of a single specific conformation. In this project, ethanol precipitation has predominantly been used as this minimises losses through use of the same tube for both precipitation and resuspension in a smaller volume. It does, however, require the protein to have been frozen in the ethanol mixture and could cause conformational defects or protein degradation.

Other types of chromatography, for example size-exclusion or antibody-binding chromatography were also options. The former would have resulted in less specifically pure antigen preparations due to selection by size rather than charge and was rejected for this reason. The latter would have involved the time consuming

process of purifying $\alpha 3(\text{IV})\text{NC1}$ -specific antibody from patient sera by chromatography or buying a commercial monoclonal antibody to the $\alpha 3(\text{IV})\text{NC1}$, in sufficient quantities to enable beads to have been labelled with the purified antibody, packed into a column and to bind the recombinant $\alpha 3(\text{IV})\text{NC1}$ protein. This technique was rejected as although it would produce a very pure protein preparation, the time involved to produce the column was not viable in a project with a very limited time frame. It would also have required a significant amount of Goodpasture patient sera; this is a scarce and valuable resource as it has to be obtained prior to immunosuppressive treatment so the technique was not deemed economical in either time or reagents.

An alternative method of purifying the protein includes extracting from an acrylamide gel. This method separates the protein by size within the gel matrix so the specific gel band containing the protein can be isolated. The gel can then be denatured and the protein extracted. This method resulted in a large amount of loss and was not suitable for use on bands containing very little protein. Certainly, bands that contained so little protein they were undetectable by coomassie staining, proved impossible to purify in this way (data not shown). The actual extraction method also required some harsh chemicals (including acetonitrile and iodoacetamide) and resulted in a lyophilised protein preparation. This method is more suitable for protein fragments undergoing mass spectroscopy and it proved very difficult to isolate sufficient protein for any experiments.

Overall, producing the $\alpha 3(\text{IV})\text{NC1}$ protein in transformed bacteria provided the greatest quantities of protein and this was able to bind antibodies in Goodpasture patient sera when immobilised on nitrocellulose membranes. The protein isolated

from human tissue was most likely in as close to the native conformation as possible, but this was very scarce and when purified resulted in a very low concentration and was insufficient for use in multiple experiments. The final method, transfection of eukaryotic cells to secrete the protein, was unsuccessful. Had it been successful, it would have most likely produced protein at concentrations greater than the protein extracted from human tissue, but lower than the concentrations produced by the recombinant bacteria. However, this would be more likely to be in a conformation similar to the native protein. None of these methods would produce the protein in exactly the same conformation as would exist *in situ* in tissue, because of the close interactions between the $\alpha 3(\text{IV})\text{NC1}$ domain and the NC1 domains of the other two α chains in the whole type IV collagen chain, $\alpha 4(\text{IV})\text{NC1}$ and $\alpha 5(\text{IV})\text{NC1}$. These effectively wrap around each other so removal of one chain or separation of the three would result in the isolated protein folding differently to protect any hydrophobic regions within the exposed protein.

To summarise, three methods of producing the Goodpasture antigen ($\alpha 3(\text{IV})\text{NC1}$) in sufficient quantities for experimental purposes were explored: bacterial recombinant expression systems, extraction of protein from human tissue and a mammalian recombinant protein expression system. Of these, extraction of the protein from human tissue produced a very low yield of protein and was only used in very limited experiments described in Chapter 7, while preliminary transformation experiments using the mammalian recombinant protein expression system did not yield measurable protein. However, the bacterial recombinant system produced the greatest yield of $\alpha 3(\text{IV})\text{NC1}$ and this was subsequently used for the majority of the experiments described within this thesis.

Chapter 6 - $\alpha 3$ wild type mouse responses to recombinant

human $\alpha 3$

6.1. – Introduction

Experiments described in this chapter were designed to examine tolerance to $\alpha 3(\text{IV})\text{NC1}$ in mice. Work in man has indicated that T cells specific for $\alpha 3(\text{IV})\text{NC1}$ exist in healthy individuals, but in health they either do not ‘see’ (are ignorant of) endogenous $\alpha 3(\text{IV})\text{NC1}$, or more likely they respond with a regulatory profile. It was hypothesised that in the latter but not the former case, immunisation with a sequence altered form of $\alpha 3(\text{IV})\text{NC1}$ would not yield a T cell response as any T cells responding to the altered part of the molecule would be subject to regulation by T cells recognising the greater proportion of self $\alpha 3(\text{IV})\text{NC1}$. To examine this hypothesis, DR15-expressing $\alpha 3(\text{IV})\text{NC1}$ -wild type mice were immunised with human $\alpha 3(\text{IV})\text{NC1}$ emulsified with strong adjuvant. The mouse and human forms of $\alpha 3(\text{IV})\text{NC1}$ are 92% sequence homologous (244) so it was predicted that immunization should yield no response at all if regulation was dominant, or a T cell response directed against the few areas of sequence dissimilarity if regulation was not dominant.

There are several ways to test T cell responses to antigen. These can include directly measuring T cell proliferation by determining the level of incorporation of tritiated thymidine (245). A more recent development is to measure uptake of

bromodeoxyuridine (BrdU), a thymidine analogue, which can then be detected by specific antibodies and the concentrations determined by spectrophotometry (246). There are also indirect (in that it does not allow for direct quantitation of activation of cells by determining proliferation and clonal expansion) ways of detecting T cell responses by measuring cytokine release in response to stimulation, for example IL-2, a cytokine released by stimulated T cells to promote proliferation (247), and interferon γ (IFN γ) which stimulates cytolytic T cell activity (248).

In order to characterise T cell responses in mice, a classical method to determine T cell proliferative responses was used. T cells were extracted and stimulated and the level of response by the T cell was determined by uptake of tritiated-thymidine into the expanding T cell population. This method was used partly for convenience – all reagents and equipment were within easy reach of the laboratory – and partly because other alternatives would have required more setting up and equipment not available to the lab at the time.

Lymphocyte stimulation assays have been used to determine T cell proliferative responses for many years, initially to determine the effects of different molecules on the immune system *in vitro*, and subsequently for more specific purposes. One publication of interest to me was the work of Abdel-Nour *et al.*, who derived T cell lines from the synovial joint fluid of patients with rheumatoid arthritis. These cell lines were then used to characterise the immune responses to collagen II, in the presence or absence of EBV transformed B cells. This work suggested that the synovial fluid itself contained a collagen II fragment that could only be presented by B cells, and that autologous B cells were also able to present the stimulatory fragment (249). The experiments described within this thesis were designed to

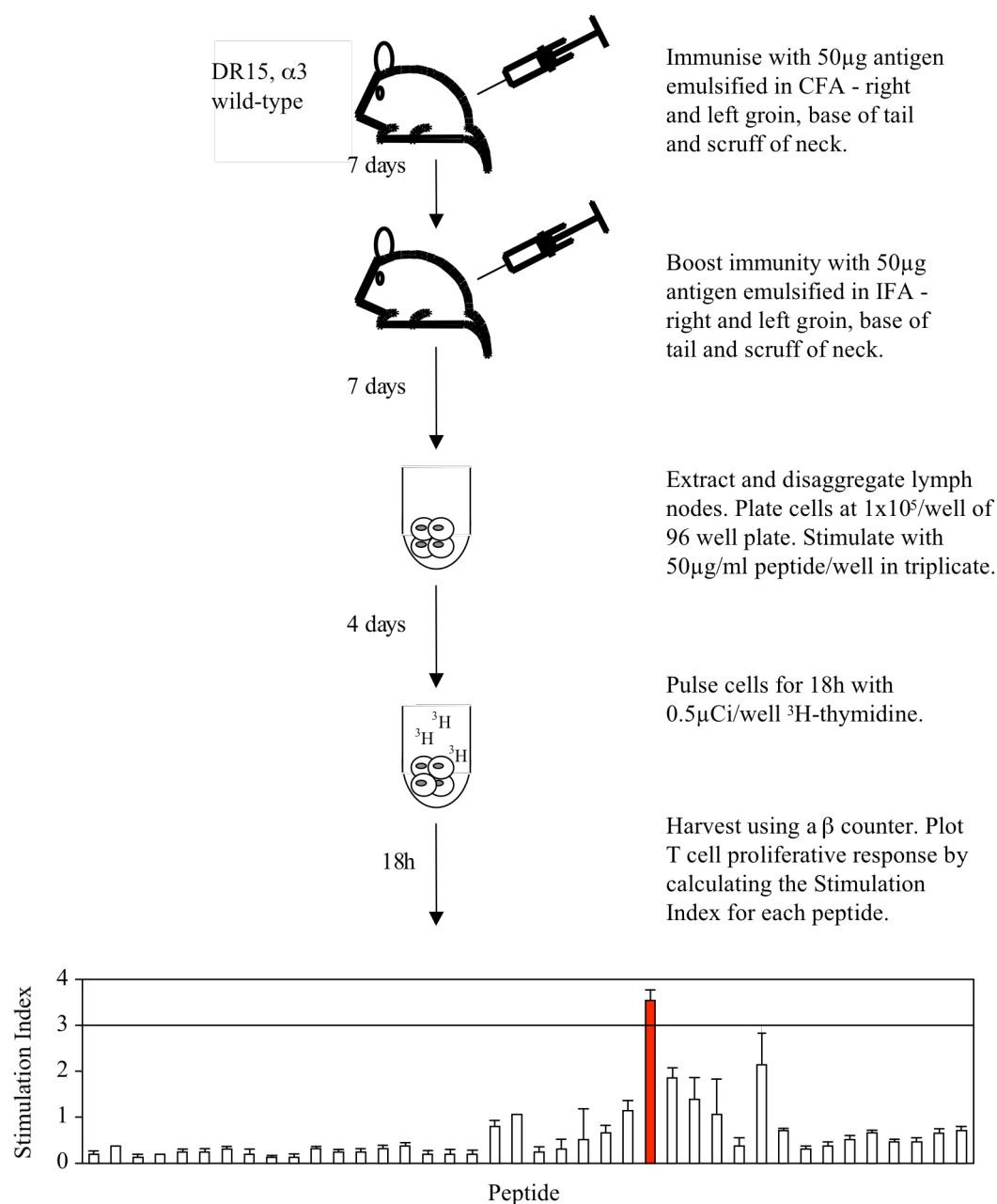
determine whether any specific fragments of the Goodpasture autoantigen were presented by B cells and if so, which induced the greatest T cell response. The work by Abdel-Nour *et al.* proved useful in determining how to undertake certain practical aspects of the project.

6.2. - Results

6.2.1. – Immune responses to $\alpha 3$ peptides in HLA DR15 positive mice

The overall experimental design used in this thesis is illustrated in Figure 6.2.1. and described in full in Chapter 2. In brief, the procedure involved emulsifying the antigen in complete Freund's adjuvant (CFA) and administering the solution to the mouse in subcutaneous injections. This was repeated a week later using antigen emulsified in incomplete Freund's adjuvant (250), before culling the animal after another week. Lymph nodes were extracted and disaggregated, the resultant cells were washed well with media before being counted and set up against a panel of overlapping 15mer peptides encompassing the entire sequence of the $\alpha 3(\text{IV})\text{NC1}$ domain. After 4 days, the cells were pulsed with tritiated thymidine for 18h before harvesting and being read on a β -counter. This data was generated as calculated counts per minute (ccpm), but this neither normalises the data nor makes it directly comparable with other experiments of the same design. In order to compare the results from different mice, the data needed to be converted into a form that is comparable between samples. The calculation for stimulation index effectively

Figure 6.2.1. – Schematic of mouse experimental design



normalises the data as it takes into account both the background radiation levels in media and unstimulated cells:

$$SI = \frac{(\text{mean ccpm sample} - \text{mean ccpm media only})}{(\text{mean ccpm unstimulated cells} - \text{mean ccpm media only})}$$

This equation gives a ratio value and, as such, enables direct comparison between samples in individual mice, for example the effect of peptide $\alpha 3(\text{IV})\text{NC1 136-150}$ in each mouse. In published experiments, there is a threshold level set that is a level, chosen as significant, of stimulation within that sample, compared to background. This is usually 2.5 or 3, for these experiments the threshold was set at 3 and is marked with a thick black line on all data presented in the form of a stimulation index. This threshold translates to the point at which there is a 3-fold increase in T cell proliferation above background.

Data sets are shown for all of the 10 $\alpha 3\text{WT}$ mice studied. There is no significant immune response to any of the overlapping peptide sequences. Figures 6.2.2A-J show the peptide stimulant on the X axis, while the calculated stimulation index is plotted, for each peptide, on the Y axis. Although there is peptide to peptide variation, there are minimal responses to whole $\alpha 3$ (example shown in Figure 6.2.3A) and an expected high response to the T cell mitogen concalavalin A (example shown in Figure 6.2.3B). Ovalbumin was used as a negative control for recall responses – the T cells would not have “seen” fragments of the ovalbumin molecule as it is foreign to mice. Therefore, the T cells should not proliferate in

response to it. Overall, there is no consistent response with a stimulation index greater than 3 to any of the overlapping $\alpha 3$ (IV)NC1 peptides.

6.2.2. – Summary of data from $\alpha 3$ immunised DR15 positive mice

Figure 6.2.4. shows a chart in which the T cell responses to each $\alpha 3$ (IV)NC1 peptide of all 10 mice have been averaged. Taking the minimum threshold for immune response to be a stimulation index of 3, even whole antigen barely reaches the value. The positive control concalavalin A (conA, a T cell mitogen and lectin derived from *Canavalia ensiformis* or the Jack bean) usually produced a response measured as a stimulation index of more than 100 (7/10). This shows that the T cells from these mice were able to proliferate, the lack of proliferative response to individual $\alpha 3$ (IV)NC1 domain peptides is indicative of a lack of overall immune response to the $\alpha 3$. This could be due to the cells requiring a combination of peptides for stimulation, so two different peptides stimulate two different cells that can then proliferate, resulting in a varied T cell population capable of responses to more than one specific epitope on an antigen. Alternatively, this could be due to a single cell being stimulated by more than one peptide (epitope spreading). There were limited responses seen to whole recombinant antigen, and these could be due to a bacterial contaminant in the rho3 preparation used to immunise the mouse and then restimulate the cells. Of these scenarios, the latter is more likely, especially as data discussed in Chapter 5 (shown in Figure 5.2.1B) shows that the preparation contained low level contamination with presumed bacterial protein. This conclusion is also somewhat supported by the lack of immune response to $\alpha 3$ (IV)NC1 peptides.

However, the recombinant antigen preparation used for all the experiments described within this thesis was maintained in 8M urea solution. This solution without antigen could be incredibly toxic to cells, so it was decided to use a titration curve to determine the possible impact of the urea in cell culture. To do this, T cells were isolated from a naïve DR15 α 3WT mouse and set up with conA, or conA plus various concentrations of urea (0.0188M, 0.188M and 1.88M). The data obtained are shown both as uptake of tritiated thymidine (Figure 6.2.5A) and expressed as a stimulation index (Figure 6.2.5B). This work showed that a relatively small amount of urea (0.0188M, the final concentration with the cells if 10 μ g recombinant α 3 solution were to be added to the well) had no significant impact on T cell proliferation in response to conA. The difference in proliferative responses was very significant in the presence of 0.188M urea – the concentration with the cells upon addition of 100 μ g recombinant α 3. As a result of these data, it was decided to use 50 μ g α 3 as a control within the T cell recall response assays, since previous work had shown that T cells were unable to respond to as little as 10 μ g recombinant α 3 (L Henderson, personal communication).

Figure 6.2.2. – Stimulation profiles from WT mice immunised with rh α 3, tested in vitro against overlapping peptide fragments of human α 3(IV)NC1.

Figures A-J show the data obtained from the T cell proliferative responses of 10 α 3WT DR15-positive mice, in response to overlapping 15mers of the α 3(IV)NC1 molecule, expressed as a stimulation index. The threshold at which there is significant stimulation above background, a stimulation index of 3, is marked on each chart with a bold horizontal black line. Each colour denotes a separate animal.

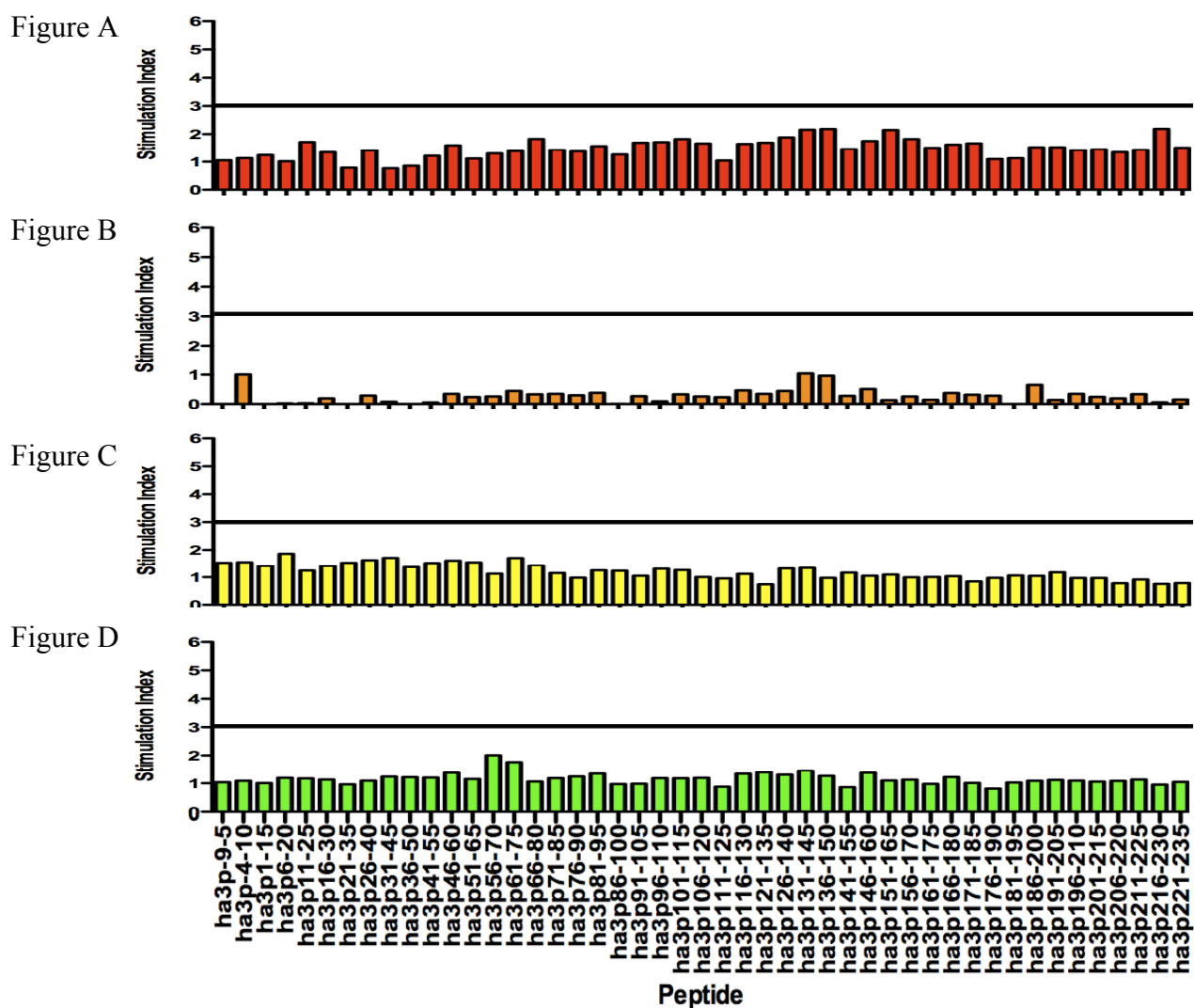


Figure 6.2.2. – Stimulation profiles from WT mice immunised with rh α 3, tested in vitro against overlapping fragments of human α 3(IV)NC1.

Figure E

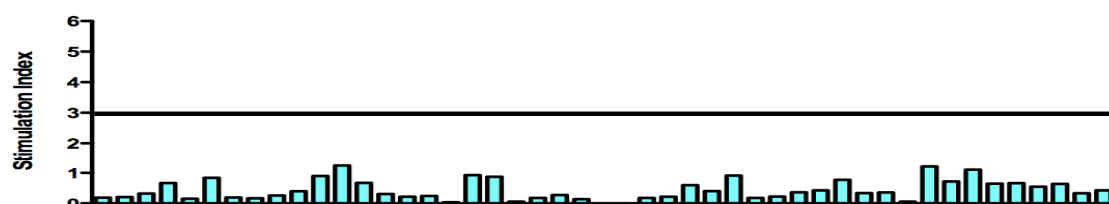


Figure F



Figure G

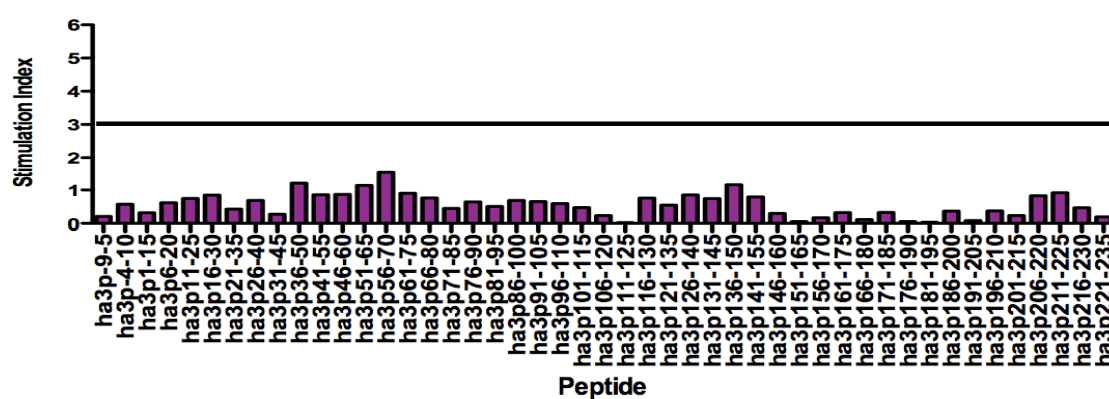


Figure 6.2.2. – Plots showing the stimulation index for each peptide and for each $\alpha 3WT$ mouse

Figure H

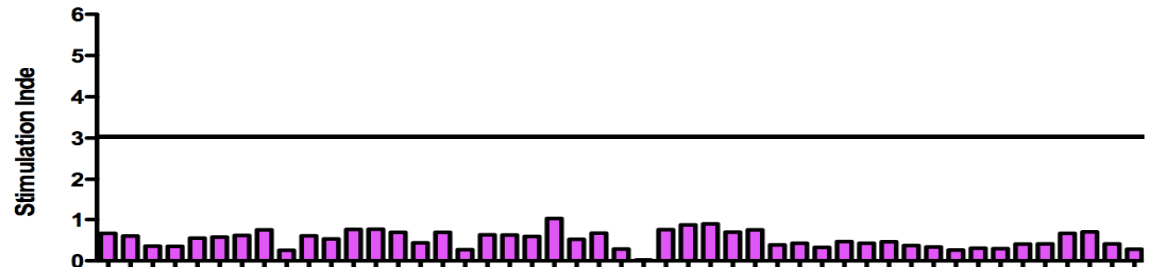


Figure I

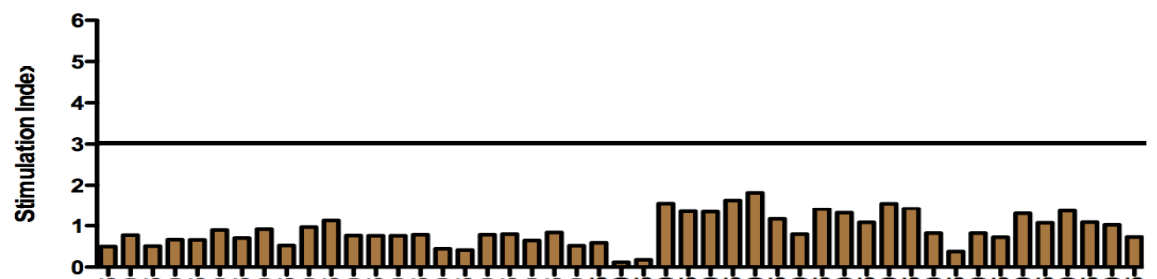


Figure J

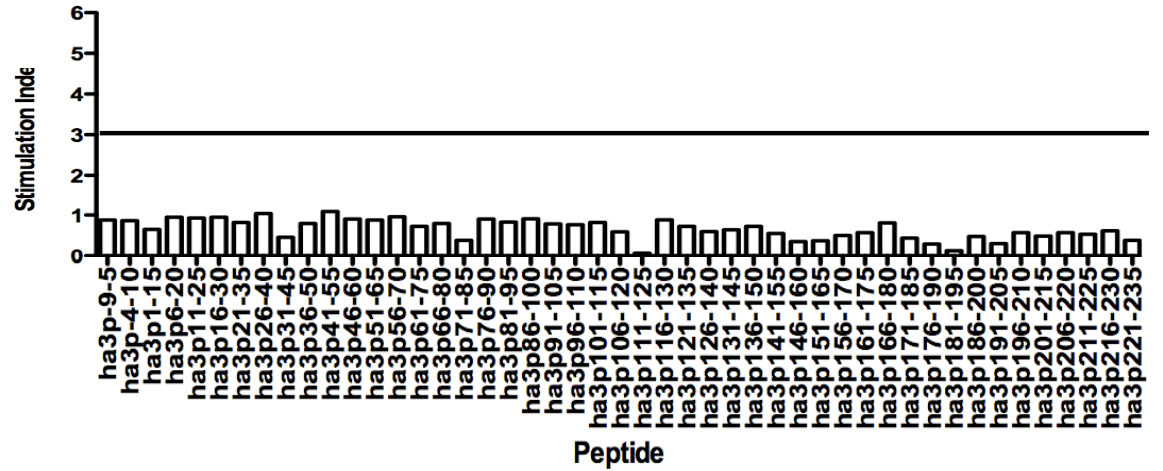


Figure 6.2.3. – Example plots showing T cell responses to control stimulants.

These data are examples of the controls included within each experiment (in this instance, n=1). Figure 6.2.3A shows example plots (calculated as stimulation index) of the negative control ovalbumin and whole recombinant $\alpha 3(\text{IV})\text{NC1}$, illustrating that there is no T cell proliferative response to these proteins. Figure 6.2.3B illustrates example plots of ovalbumin compared with conA. These plots show that conA clearly stimulates the T cells to proliferate, compared with ovalbumin. As with previous stimulation index data, the threshold of 3 is marked with a bold black line.

Figure A

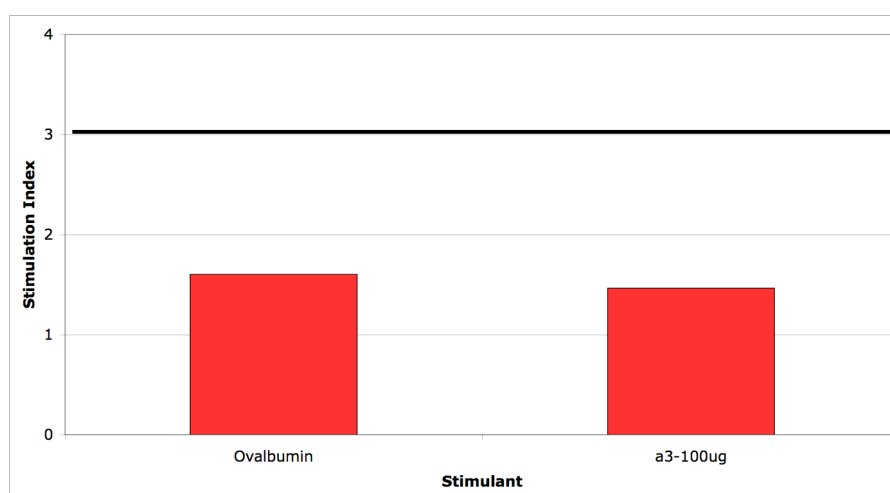


Figure B

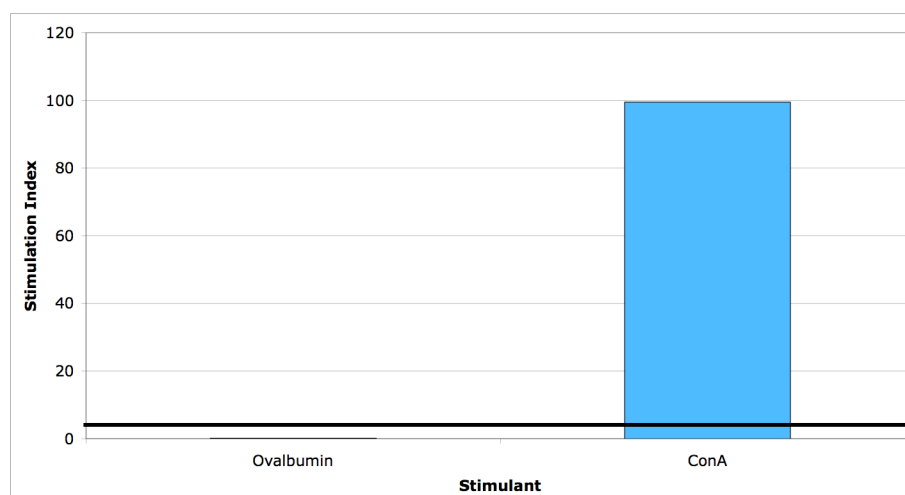


Figure 6.2.4. – Summary chart plotting peptide against mean stimulation index for all mice studied.

This chart shows the average SI obtained from 10 mice for each peptide. As can be seen, none of the peptides used in the restimulation assay resulted in a proliferative response with a stimulation index greater than 3 (marked with a bold black line). The error bars show the standard deviation above and below the mean for each peptide.

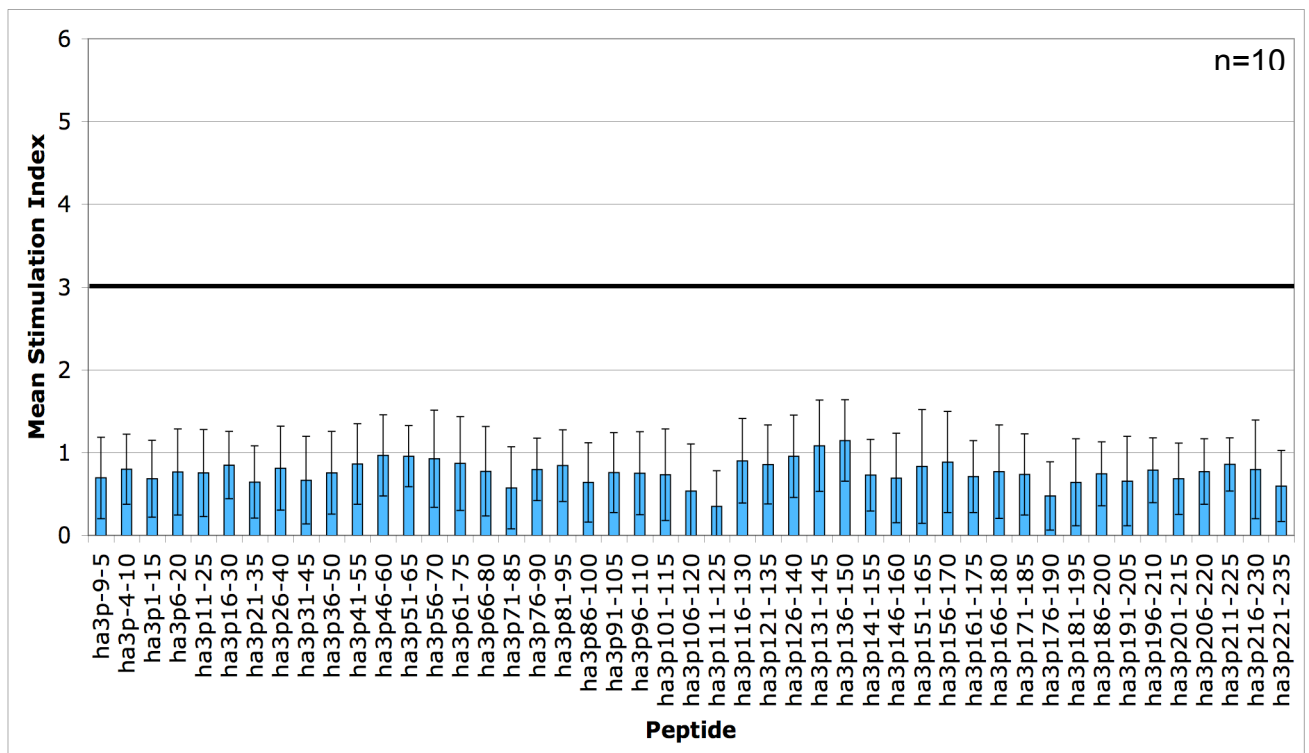


Figure 6.2.5. – T cell proliferative responses to conA in the presence of urea

T cells from $\alpha 3$ WT mice were stimulated with conA in the presence of increasing concentrations of urea (corresponding to 10, 100 and 1000 μ g recombinant human $\alpha 3$ (IV)NC1. Figure 6.2.5A shows proliferative responses expressed as uptake of tritiated thymidine (ccpm), while Figure 6.2.5B shows the same data expressed as a stimulation index. As with previously shown data, a stimulation index of 3 is marked with a bold, black line.

Figure A

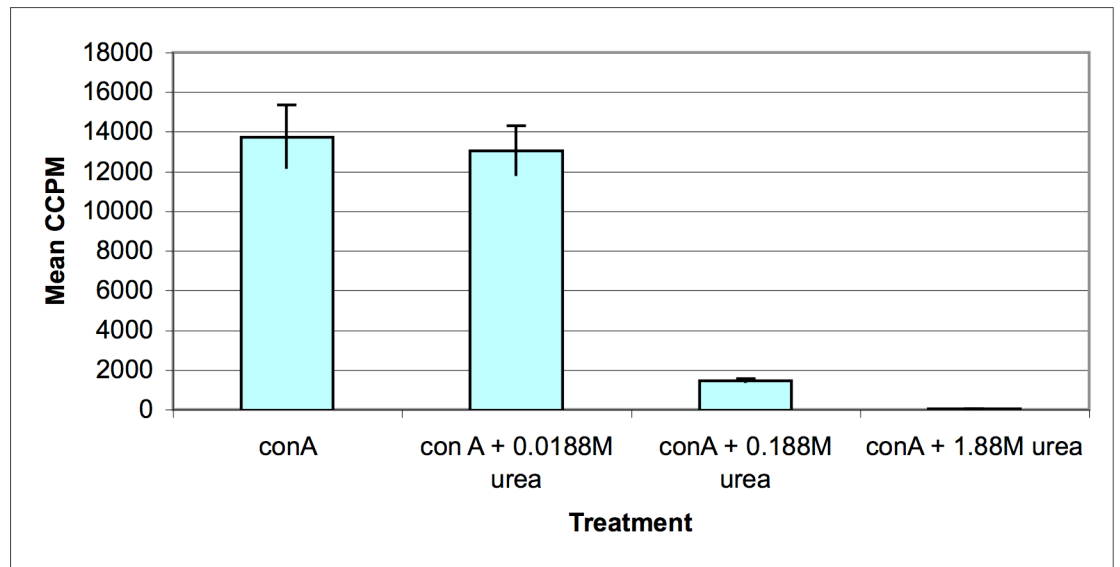
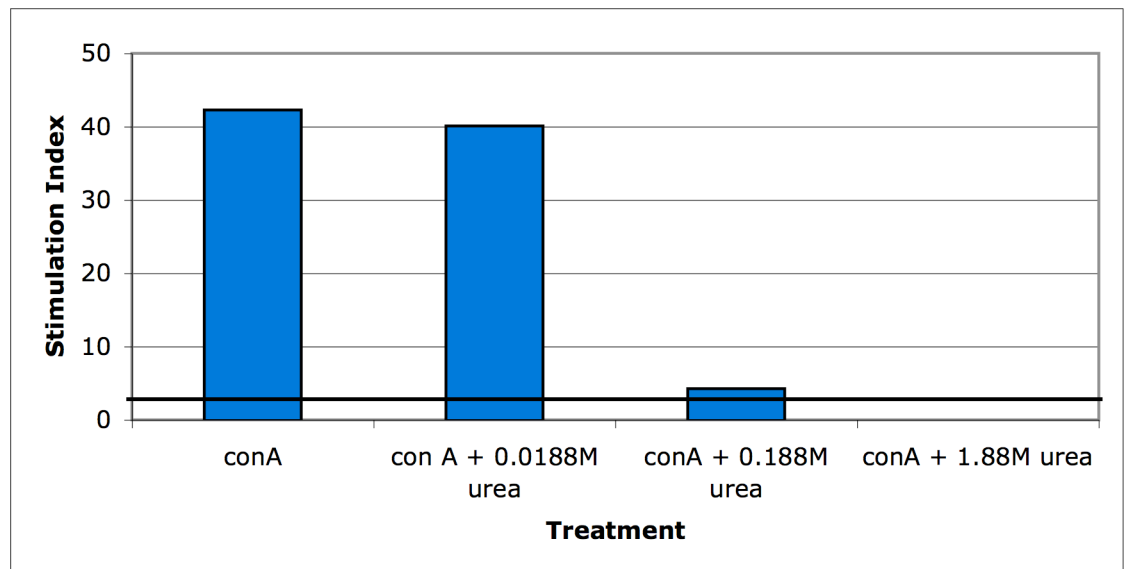


Figure B



6.2.3. – DR15 positive mice do generate normal immune responses

Since these experiments showed a general lack of reactivity to $\alpha 3$, it was decided to test the experimental system to an antigen expected to evoke T cell responses, in order to rule out any methodological explanations for the apparent non-responsiveness of the mice. To do this, the same experimental design as for testing the immune responses to $\alpha 3(\text{IV})\text{NC1}$ was used, but mouse class II null, HLA DR15 positive, $\alpha 3\text{WT}$ mice were immunised with ovalbumin (approximately 38% sequence homology with mouse albumin). The cells were extracted from the lymph nodes using the same technique as for the $\alpha 3(\text{IV})\text{NC1}$ responses, but set up against a more limited selection of stimulants.

For this experiment, Con A was used as a positive control, the peptide sequence known as AS345 ($\alpha 3(\text{IV})\text{NC1}$ 65- 82 with an additional serine at the start of the selected sequence) was used as a negative control, ovalbumin was tested and $\text{rh}\alpha 3$ was also tested at three different concentrations.

The sole response was to ovalbumin ($\text{SI} \approx 45$, $\text{N}=2$). This was surprising as Con A would be expected to produce a strong mitogenic response, but subsequent testing showed that the Con A used in these experiments was defective. A new batch tested on naïve $\alpha 3\text{WT}$ mice was found to produce good proliferative responses. This experiment was designed to test the immune responses to exogenous antigen, so the absence of a mitogenic control would only have been an problem if the mice showed no immune response to the foreign antigen.

Figure 6.2.6. – Mice immunised with ovalbumin were capable of generating an immune response.

Figure A shows recall responses of lymphocytes from mice primed by immunisation with ovalbumin (background subtracted), while Figure B shows the same data normalised and expressed as a stimulation index. In these mice, ovalbumin produced a T cell proliferative response, while $\alpha 3$ did not. Con A did not stimulate a T cell proliferative response, but it was subsequently found that the con A preparation used in this experiment had suffered freeze-thaw damage. An experiment using naïve mouse lymphocytes showed that a new batch of Con A was mitogenic (data not shown).

Figure A

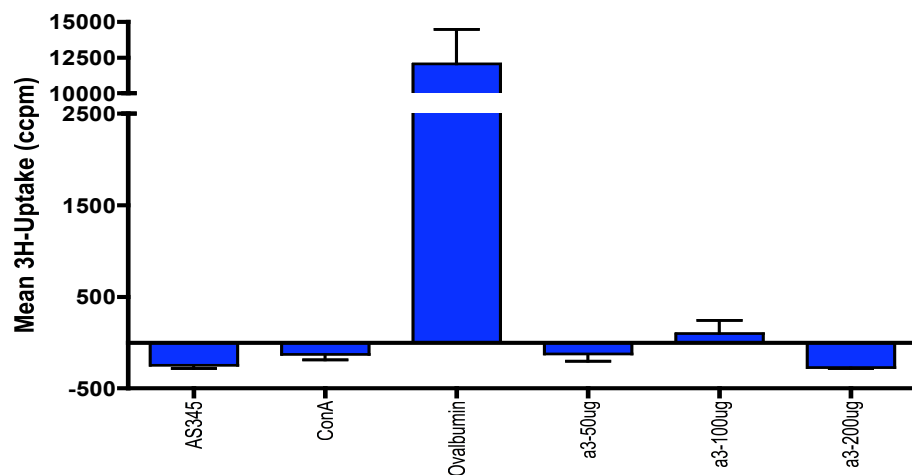


Figure B

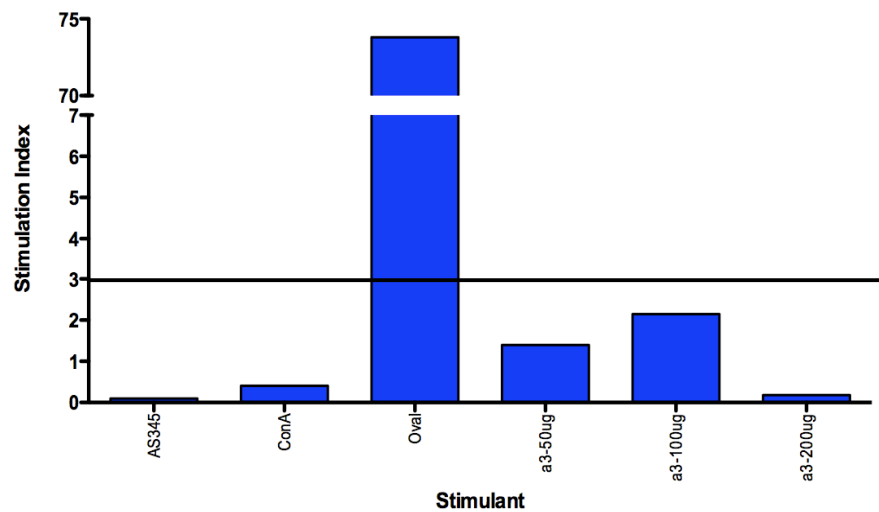


Figure 6.2.7. – Haematoxylin and eosin stained kidney sections from naïve and immunised mice.

These photos show naïve mouse kidney (Figure A), ovalbumin immunised mouse kidney (Figure B) and rh α 3 immunised mouse kidney (Figure C). As is shown, all glomeruli look normal irrespective of immunisation schedule. Images shown at x400 magnification.

Figure A – Naïve Mouse

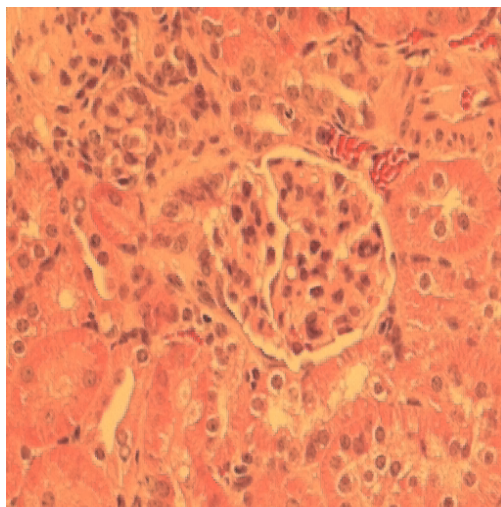


Figure B - Ovalbumin

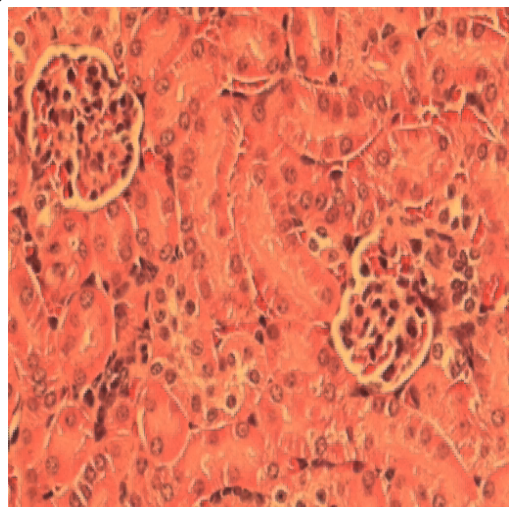
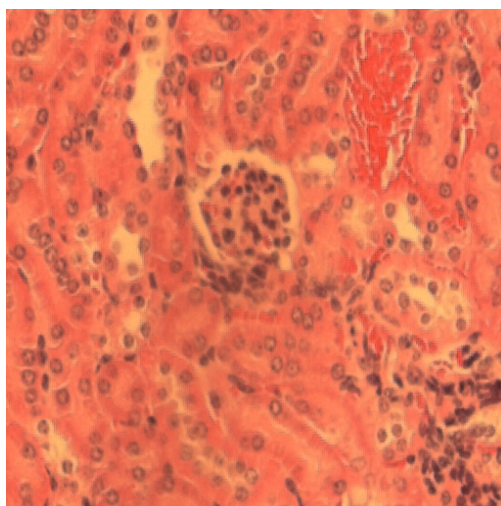


Figure C - rh α 3



6.2.4. – Comparing morphology of immunised and unimmunised kidneys

As well as testing for immune responses, evidence of morphological and potentially pathological responses to rh α 3 within the kidney itself were studied. Effectively, evidence of immune injury in the kidney was investigated as this may be more sensitive to low level responses (as observed in Goodpasture's disease in man) (251). The kidneys from each immunised mouse were harvested and fixed in 10% neutral formalin, prior to paraffin embedding and sectioning. By staining the sections with haematoxylin and eosin, the morphology of kidneys from immunised and naive control mice could be compared (Figure 6.2.7.). This data showed that kidneys harvested from immunised mice did not show any morphological changes following immunisation, when compared with kidney sections from an unimmunised mouse. However, the presence of anti-glomerular basement membrane antibodies within the sections was not investigated due to time constraints.

6.3. - Discussion

The experiments described above form two sets of data. The first set, testing the recall responses to rh α 3 showed few immune responses to the α 3(IV)NC1 peptides, while the second set of data, showing the histology of the kidney following immunisations showed no obvious pathology. Together, these suggest effective self-tolerance dominates, despite some differences between human and mouse α 3(IV)NC1 sequences (92% homology).

Despite a strongly stimulatory immunisation regime (one immunisation of antigen emulsified in complete Freund's adjuvant, followed a week later by a booster immunisation for antigen emulsified in incomplete Freund's adjuvant), mice with an intact $\alpha 3$ gene do not generate T cells that proliferate in response to any part of human $\alpha 3$ NC1 domain. The most likely explanation is tolerance mechanisms that prevent responses to mouse $\alpha 3(\text{IV})\text{NC1}$ are able to extend tolerance to human $\alpha 3(\text{IV})\text{NC1}$ despite its sequence dissimilarities. The mouse $\alpha 3(\text{IV})\text{NC1}$ domain has 92% homology with human, but this degree of homology does not prevent T cell responses to other homologues of self proteins in mice. Two such examples include collagen II and rheumatoid arthritis in which there is 95% protein homology between mouse and human protein sequences (252), and myelin basic protein and experimental autoimmune encephalomyelitis (akin to human multiple sclerosis) in which there is 92% protein homology between the mouse and human sequences (253). Since these proteins show high levels of homology but can still induce autoimmunity, it suggests that the mechanisms preventing immune responses to $\alpha 3(\text{IV})\text{NC1}$ are regulatory, rather than owing to sequence homology.

For the experiments described, the peptides used were synthetic and arrived lyophilised and were stored in DMSO prior to use. If the immune responses seen in the wells containing whole antigen were due to antigen recognition, the responses would also occur in wells containing pure fragments of antigen if all possible epitopes were represented. Instead, because the peptides were not produced by bacteria, they are free from any additional bacterial proteins that could induce an immune response in the T cells. This suggests that the T cell response to whole $\alpha 3(\text{IV})\text{NC1}$ may be due to bacterial contaminants within the preparation.

In addition, the different conformation of the immunising antigen (rh α 3) compared with native human antigen (hu α 3) is an issue. The experimental design does not differentiate between B cell epitopes, which are conformation dependent. While my experiment does rely on there being APCs present in the lymph nodes to process and present the antigen from the surrounding media *in vitro*, these APCs do not have to be B cells and are mostly likely to be a mixture of B cells, dendritic cells and macrophages, owing to the relatively crude preparation of cells used as the source material. This implies that the specific processing events vary and that different antigenic fragments are presented.

The main result seen from my experiments as described above, is that immunisation with rh α 3(IV)NC1 in mice expressing mouse α 3(IV), does not result in a measurable immune response to α 3(IV)NC1 peptides. Importantly, this is not due to a general inability of the mice to generate a T-cell mediated immune response, as they are capable of generating a response to a foreign antigen ovalbumin (only 38% sequence homology found between ovalbumin and mouse albumin) (section 6.2.3.) and also to intact α 3(IV)NC1 bacterial products. This supports the hypothesis that the deficit of immune response may be due to tolerance mechanisms.

This overall conclusion from the work in this chapter is not entirely unexpected, but it was anticipated that there would be some response to less homologous regions of the α 3(IV)NC1 protein. Other experiments have utilised ovalbumin (ovalbumin is foreign to mice and has 60% protein sequence homology (BLAST) with serpin B1, and 38% homology with mouse albumin) and tested immune responses to the protein compared with bovine serum albumin and hen egg lysosomes (254), and have also used ovalbumin as a surface marker for targeted

destruction of solid tumours (247). In comparison to ovalbumin, there is a higher percentage of homology between the human and mouse $\alpha 3(\text{IV})\text{NC1}$ (92%) (244) so tolerance to homologous regions would be expected, however, even in areas with less homology, there was no measurable immune response. This suggests that tolerance is induced to selected $\alpha 3(\text{IV})$ peptide fragments in the thymus, or within the periphery, which happen to be homologous with those derived from the human $\alpha 3(\text{IV})\text{NC1}$ sequence. Alternative explanations include that tolerance is induced to the complete $\alpha 3(\text{IV})\text{NC1}$ protein, irrespective of peptide presentation or that tolerance is strictly regulated by T cells capable of recognising similar epitopes from both man and mouse $\alpha 3(\text{IV})\text{NC1}$. Of these hypotheses, the former is more likely.

Many previous experiments have involved looking at peptide presentation and T cell stimulation in transgenic specific gene knock-out animals, as a way of circumventing tolerance. This system ensures that the animal effectively treats the protein as a foreign antigen. Other groups have approached studying tolerance by trying to break tolerance to a specific protein. Breaking tolerance is notoriously difficult. There is a lot of research ongoing to find ways to break tolerance and enable tumour destruction or conversely, to induce tolerance in autoimmune disease or to prevent transplant rejection. Methods tested for their ability to break tolerance have included even more severe immunisation regimes, immunising with anti-CD40 to increase CTL responses and immunising with sub-dominant peptides to which tolerance may be less secure (section 1.4.) (122). Grossmann *et al.* compared these three methods of breaking immunological tolerance to prostate-associated antigens (PAA) in prostate cancer, and found that while the first two resulted in immune responses, neither broke tolerance, but the last both induced an immune response and

broke tolerance. An equivalent experiment to study subdominant immune responses would have been to immunise the DR15 positive $\alpha 3(\text{IV})\text{NC1}$ WT mice with $\alpha 3(\text{IV})\text{NC1}$ peptides, isolate the T cells and assess the resultant immune responses. The work by Grossman *et al.* suggests that not all fragments of an antigen are presented in the thymus and further supports the hypothesis that tolerance is induced to the major naturally processed and immunodominant peptides presented by thymic epithelial cells (136). In this context, only the immunodominant peptides from common proteins are presented to T cells during their maturation process, so tolerance is not induced to subdominantly presented peptides and therefore the subdominant peptides are a viable proposition for breaking tolerance to a specific protein. This approach is currently under investigation as a way to induce an immune response to various cancers, all of which have some immune privileges (255) so are unable to be detected by the immune system. This has been shown in a disparate group of cancers including prostate, leukaemia and some solid lymphatic tumours (122, 256, 257). Despite investigating how to break tolerance and enable the immune system to destroy cancers, this is a highly unnatural sequence of events. None of the methods described for potentially breaking tolerance in laboratory models is likely to be a cause of autoimmunity in humans. These methods could, however, lead to valuable information regarding the mechanisms that implement tolerance. Two very specific and contrasting uses for this information would include how to up-regulate tolerance in order to prevent transplant rejection, or how to specifically down-regulate the effect of the same mechanisms to produce an immune response to cancer without down-regulating tolerance as a whole.

The aim of this chapter was to study specificity of responses to $\alpha 3$ in presence of tolerance, although tolerance may be incomplete due to sequence differences (human vs mouse). In this respect, it was determined that despite differences in the $\alpha 3(\text{IV})\text{NC1}$ protein sequence between mice and humans, the overall tolerance to mouse $\alpha 3(\text{IV})\text{NC1}$ is sufficient to prevent T cell proliferative responses to human $\alpha 3(\text{IV})\text{NC1}$. The differences between the human and mouse $\alpha 3(\text{IV})\text{NC1}$ sequences are compared in Figure 6.3.1.. The mouse and human $\alpha 3(\text{IV})\text{NC1}$ sequences are very similar (92% homology (244)). Perhaps a better way to compare sequences with regard to their capacity to evoke T cell responses is to compare their content of sequences with higher affinity for HLA class II molecules that might act as T cell epitopes.

These results suggest that there is secure tolerance to constitutively presented or most abundantly presented peptides. Additional work undertaken within the laboratory used a different peptide, $\alpha 3(\text{IV})\text{NC1}$ 65-82, shown to occasionally elicit an immune response in Goodpasture patients (*ex vivo*), to immunise DR15 $\alpha 3$ KO mice. These experiments were unable to detect a T cell proliferative response to this peptide (data not shown), despite showing a response to $\alpha 3(\text{IV})\text{NC1}$ 136-150. This could be due to the peptide selected ($\alpha 3(\text{IV})\text{NC1}$ 65-82) being only able to generate a very low immune response generally. Using an online algorithm, the predicted IC_{50} for this peptide is 9844.44nM, from input of the sequence into the website,

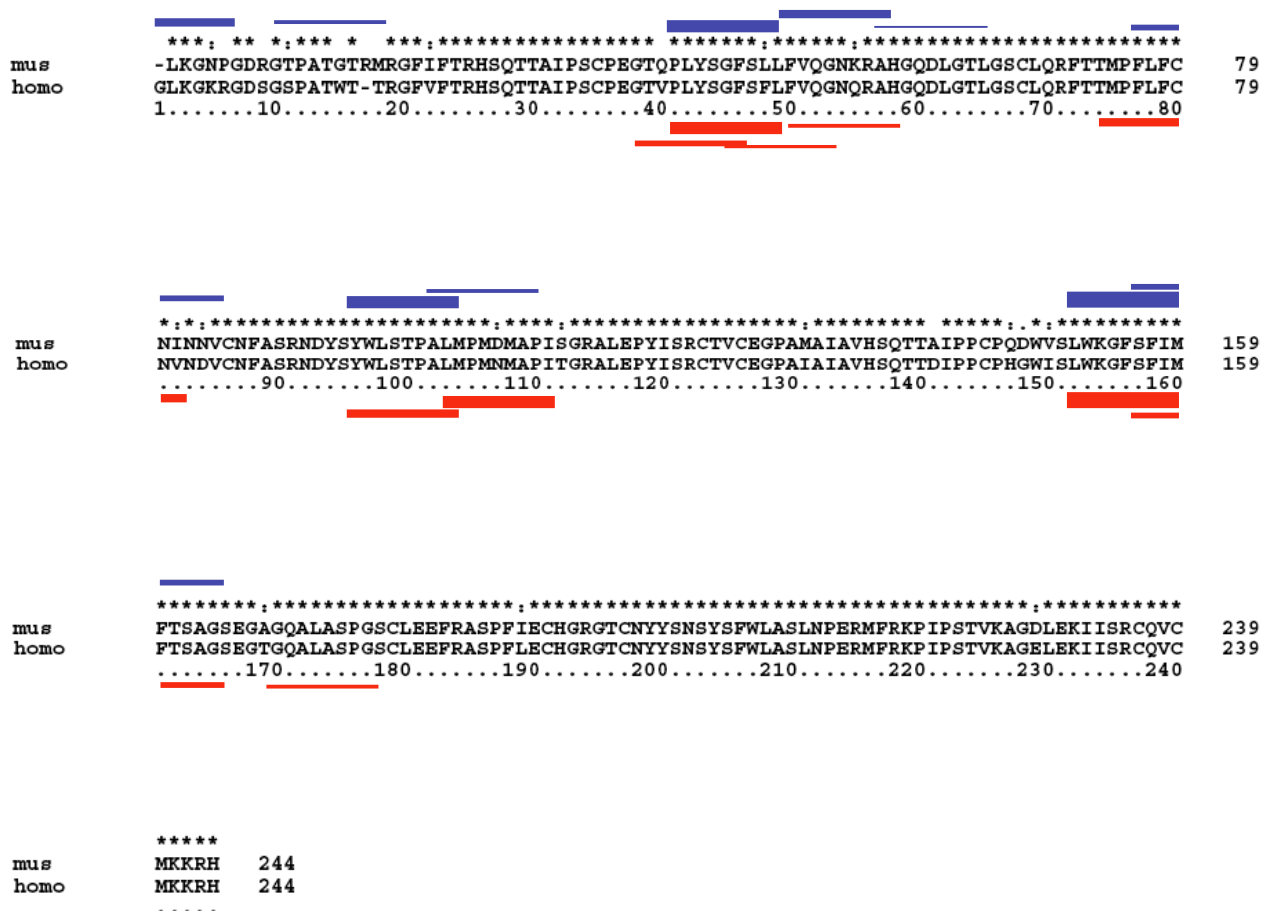
http://tools.immuneepitope.org/tools/matrix/iedb_input?matrixClass=II.

This is a tool that analyses the sequence for binding capacity to the specific MHC class II molecule and can be used to predict the concentration at which 50% of the MHC class II molecules are occupied (or inhibited) by the peptide fragment, the

IC₅₀ (258-260). Other peptides from the $\alpha 3(\text{IV})\text{NC1}$ sequence may produce a greater response and prove more suitable for use in breaking tolerance. It is not entirely surprising that the prominent HLA-DR15 epitopes within the sequences are not only similar, but overlap between the sequences. The $\alpha 3(\text{IV})\text{NC1}$ protein sequences illustrated (Figure 6.3.1.) were aligned using clustalX software (261, 262) and epitope binding sequences calculated using the online algorithm described above (258-260). The epitopes show marked similarity between mouse and human binding regions, but also illustrates the overlap within the regions and show areas of the protein with multiple binding epitopes with different binding affinities for HLA-DR15. The Figure shows the top 10 binding epitopes for HLA-DR15 along the sequence of the $\alpha 3(\text{IV})\text{NC1}$ proteins, estimated using the online HLA binding algorithm described previously, both for mouse and human proteins, enabling comparison between the two. These similarities support the data shown in within this chapter, whereby mice with tolerance to murine $\alpha 3(\text{IV})\text{NC1}$ did not produce immune responses to human $\alpha 3(\text{IV})\text{NC1}$. They also support the finding the $\alpha 3(\text{IV})\text{NC1}$ 136-150 is the immunodominant peptide as both the murine and human epitope with highest binding affinity bind within this region, while there are additional epitopes nearby.

Figure 6.3.1. – HLA-DR15 binding motifs on mouse and human $\alpha 3(IV)NC1$ protein sequences

This Figure shows the protein sequences of mouse (top, mus) and human (bottom, homo) $\alpha 3(IV)NC1$, aligned using clustal software. Conserved residues are marked *, residues marked : have similar properties and those marked . are less similar. In addition, the 10 epitopes with the highest affinity for HLA-DR15 from each sequence are marked with lines highlighting the specific sequence (blue, top shows mouse epitopes; red, bottom shows human epitopes). These lines are thickest where there is highest binding affinity ($IC_{50} = 2.46nM$ for both mouse and human epitopes). As can be seen, there is considerable overlap for DR15 specific binding epitopes, both within mouse and human, and between the two sequences.



In this thesis, the classical experimental design of immunisation, boost, recall response has been maintained, but other work has exploited new technology to improve the overall safety of the recall responses assay. With concerns about the future impact on both the researchers and the surrounding environment, alternatives to radioactivity have been sought. Of particular interest is the substance bromodeoxyuridine or BrdU, a fluorescent uridine analogue that can be incorporated into cells in a similar way to tritiated thymidine. BrdU can also be administered directly into cells or animals, enabling direct imaging of specific events and regions, conferring a significant advantage to the molecule over the more traditional radioisotopes. Again, this has been used in classical experiments to determine specific immune responses to processed proteins in disease conditions, for example the effect of HSP65 in Takayasu's Arteritis (263), but has also been used to label cells for flow cytometry, and to track proteins and cell types *in vivo*. In doing so, it enabled the determination of key regulatory molecules for antigen-induced T cell activation and proliferation, that also have implications in suppressing tumorigenesis (264). Although BrdU was tested for its suitability for use during this project, the facilities for measuring the output using tritium gave more consistent data, hence the use of tritiated thymidine throughout this project.

More recent work by Ghaffari *et al.* (265) has focussed on different aspects of the lymphocyte stimulation assay, and involved trying to determine *in vitro* the T cell response to a novel antigen, rabies. This was mainly carried out *in vitro* and the experiments involved immunising healthy volunteers with rabies vaccine, before isolating a sample of peripheral blood mononuclear cells and testing the immune

responses over a time course against phytohaemagglutinin (negative control), tetanus toxoid (positive control as most people in the UK are immunised against tetanus as standard) and the rabies antigen. This work, while inconclusive about the actual mechanisms underlying the responses to a viral antigen, did however illustrate that the rabies vaccine has potential applications in determining the degree of immunosuppression in affected individuals (265). Other work has studied the impact of macrophages from within the lung on T cells, trying to determine how lung homeostasis is maintained as this system is notorious for varied T cell responses. On the one hand, it has been shown that there is T cell suppression by alveolar macrophages in young infants (266). On the other hand, the lung is inextricably linked with the outside environment and comes in contact with massive numbers of potential pathogens daily (267). This variability in immune response and T cell suppression versus activation can be partially explained by the difference in macrophage phenotype as there are both activating and suppressive phenotypes and this is mirrored by the cytokines they produce. The two phenotypes work together to neutralise threat, and to prevent a systemic immune response to an already neutralised pathogen (6).

To summarise, the work in this chapter suggests that tolerance mechanisms within the DR15-expressing, $\alpha 3(\text{IV})\text{NC1}$ wild-type mice are robust when challenged by a strongly stimulatory regime. This was despite immunising with human $\alpha 3(\text{IV})\text{NC1}$, where tolerance was induced to mouse $\alpha 3(\text{IV})\text{NC1}$. The homology between the two sequences is 92%. The lack of immune responses seen to overlapping $\alpha 3(\text{IV})\text{NC1}$ peptides was not due to an overall inability to generate an immune response, as strong proliferative responses were measured to ovalbumin.

Chapter 7 – Proliferative responses to $\alpha 3$ in $\alpha 3$ null mice

7.1. - Introduction

The experiments described in this Chapter were designed to further address the nature of tolerance to $\alpha 3(\text{IV})\text{NC1}$ by determining the fine specificity of T cells responding to $\alpha 3(\text{IV})\text{NC1}$ in mice where tolerance to $\alpha 3(\text{IV})\text{NC1}$ is not present. The results would inform as to the importance of T cell deletion in tolerance to $\alpha 3(\text{IV})\text{NC1}$. Since $\alpha 3(\text{IV})\text{NC1}$ was found in the human thymus (4), it had been thought likely that at least the most abundantly presented $\alpha 3(\text{IV})\text{NC1}$ peptides were likely to stimulate deletion of cognate T cells, and the T cells in the peripheral circulation should be specific for subdominant peptides. Two lines of evidence challenged this view. First, among the peptides shown to stimulate T cells from patients with acute Goodpasture's disease were some from regions of $\alpha 3(\text{IV})\text{NC1}$ known to be very efficiently presented from biochemical elution studies (so should have been deleted). Second, the fine specificity of $\alpha 3(\text{IV})\text{NC1}$ -reactive T cells in a broad panel of healthy individuals showed remarkable similarity in their responsiveness to a number of $\alpha 3(\text{IV})\text{NC1}$ peptides. This would not be expected of sub-dominant responses restricted by different HLA molecules in different individuals. These lines of evidence could be further examined by determining which epitopes within $\alpha 3(\text{IV})\text{NC1}$ were immunodominant in the context of HLA-

DR15 by immunising $\alpha 3(\text{IV})\text{NC1}$ null, DR15-expressing mice with human $\alpha 3(\text{IV})\text{NC1}$ and investigating the peptide specificity of responding T cells.

In this Chapter, although I tested the recall responses of the DR15 A3v $\alpha 3\text{KO}$ mice in the same way as I tested the recall responses of the DR15 A3v $\alpha 3\text{WT}$ mice, I also wanted to test the responses in a slightly different way both to corroborate data obtained from the recall response assay. I decided to generate hybridomas from the T cells extracted from $\alpha 3\text{KO}$ mice immunised with $\text{rh}\alpha 3$, that would enable me to test recall responses by production of IL-2. These hybridomas could then also be used for future experiments testing responses to specific peptides and peptide presentation by APCs.

Hybridomas were first created by Kohler and Milstein (268) in order to produce monoclonal antibodies. Subsequently, selection procedures exploiting the T cell I-J determinants and the suppressor T cell ability to bind antigen were developed by Tanaguchi and Miller in 1978 (269) and increased the probability of isolating a suppressor T cell hybridoma. Despite this, techniques to produce cytotoxic (CD8 positive) T cell hybridomas were not successfully utilised until 1980, by Nabholz *et al.* (270). Since then, T cell hybridomas have been used extensively in researching T cell immunology, including the determination of immunodominant responses (271-273). One particular example is in that of the Fathman lab, where hybridomas are used to study and try to quantify the methods of T cell activation (49, 274). These studies found that avidity for the antigen by the T cell receptor, the concentration of the MHC: peptide complex on the APC, or density of the T cell receptor on the T cell surface were equally important in the activation of T cells.

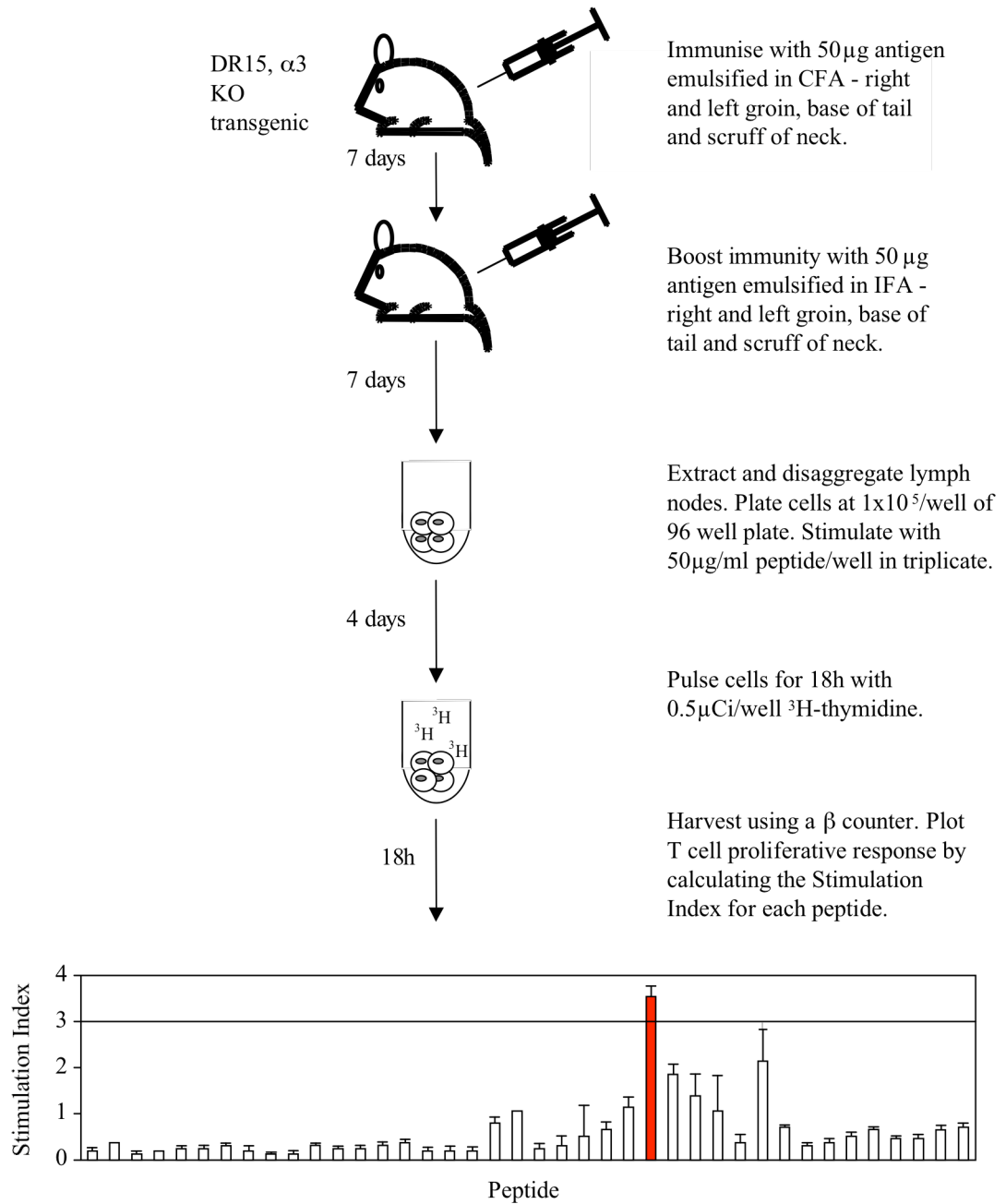
During this project, I utilised the methods published by Prof C. G. Fathman (49, 274) and by Dr J. Robinson (275) to produce CD4 positive T cell hybridomas.

7.2. - Results

As in Chapter 6, DR15-expressing mice were immunised with $\alpha 3(\text{IV})\text{NC1}$ in complete Freund's adjuvant on day 1, boosted with $\alpha 3(\text{IV})\text{NC1}$ in incomplete Freund's adjuvant on day 8, then culled on day 15. T cells were extracted from the lymph nodes and set up against a panel of overlapping peptides from the injected antigen, in a lymphocyte stimulation assay. This technique is well established, but does have limitations. The major one being that should the experiment fail for any reason (premature cell death or problems with harvesting, for example), the cells are lost and to all intents and purposes, the original animal has been wasted. An alternative way of investigating the immune response is by turning the cells into hybridomas, and this was used to test recall responses in the $\alpha 3$ null mice and to validate the data obtained from testing the recall responses in primary cells.

The experiments undertaken for this Chapter all use mice with the same genotype, mouse class II null, HLA-DR15 positive, $\alpha 3(\text{IV})\text{NC1}$ null, and used to further characterise the immune responses. These mice were immunised with rh $\alpha 3$ or hu $\alpha 3$, or immunised with rh $\alpha 3$ then the cells isolated used to produce hybridomas.

Figure 7.2.1. – Schematic of mouse experimental design



7.2.1. – Immune responses to bacterial recombinant human $\alpha 3(\text{IV})\text{NC1}$

T cells from $\alpha 3(\text{IV})\text{NC1}$ -immunised mice were harvested and their fine specificity determined by lymphocyte stimulation assays set up in the same way as in Chapter 6. It was seen that, as with $\alpha 3$ wild type mice, there were consistent responses to con A, but also to whole $\alpha 3$ (example data shown in Figure 7.2.2.). The decrease in stimulation index in the presence of higher concentrations of $\alpha 3$ is more likely to be due to the increased concentration of urea within the culture than toxicity from an increased concentration of $\alpha 3$ (urea titration shown in Figure 6.2.6.). Figure 7.2.3. shows the data obtained from 7 $\alpha 3\text{KO}$ mice, with each peptide induced response expressed as a stimulation index to enable direct comparison (calculation described in Chapter 6). The responses to peptides seen in these $\alpha 3$ null mice were different from those seen in the $\alpha 3$ WT mice. The immune response varied between individual $\alpha 3$ knockout mice, but most (5/7) of the mice responded to the peptide $\alpha 3(\text{IV})\text{NC1}$ 136-150. Other stimulatory peptides included $\alpha 3(\text{IV})\text{NC1}$ 101-115, $\alpha 3(\text{IV})\text{NC1}$ 151-165, $\alpha 3(\text{IV})\text{NC1}$ 191-205 and $\alpha 3(\text{IV})\text{NC1}$ 206-220. While these experiments did illustrate immune responses to some peptides within $\alpha 3(\text{IV})\text{NC1}$, overall these responses were still at far lower level than expected. This could be due to the $\alpha 3(\text{IV})\text{NC1}$ preparation used for immunisation being more dilute than believed. The data from all 7 mice have been combined to give a mean (shown in Figure 7.2.4). As illustrated by the error bars (standard deviation from the mean), there is considerable variation for the level of stimulation by each peptide and some of the stimulation patterns can be lost by presenting the data in this way.

7.2.2. – Immune responses to exogenous antigen

In order to check that the transgenic mice were capable of generating a normal immune response, the mice with the greatest amount of modification (ms class II null, HLA-DR15 positive, $\alpha 3$ null) were tested for their responses to a different exogenous antigen, in this case, ovalbumin. T cells were harvested, incubated *in vitro* with ovalbumin and APC, and after 1-11 days, aliquots of the T cells were pulsed with tritiated thymidine and incorporation determined at 24 hours (Figure 7.2.5.). These results showed that the optimal time point for pulsing the cells in order to detect a proliferative response to *in vitro* restimulation was on day 6 to read the plates on day 7. However, all other results of proliferation assays I have described were obtained by pulsing cultured cells with tritium on day 4 and reading the plates at day 5 following early experiments in which increased cell death was observed after day 5. This increased cell death resulted in increased difficulty in interpreting data obtained from later time points (data not shown). Therefore, these results show that the $\alpha 3$ KO mice were capable of generating an immune response to exogenous antigen, suggesting that their immune system is not especially defective.

7.2.3. – Immune responses in $\alpha 3$ null mice immunised with human

$\alpha 3(IV)NC1$

Figure 7.2.2. – Example controls from $\alpha 3$ KO T cell proliferative responses

These charts show example controls used when setting up each T cell proliferative response assay. Figure 7.2.2A shows that ovalbumin does not result in T cell proliferation but that $\alpha 3$ results in stimulation suggesting that an immune response has been successfully generated to the immunising antigen (unlike in $\alpha 3$ WT mice, Figure 6.2.3.). ConA also results in T cell proliferation (Figure 7.2.2B). A stimulation index of 3 is marked with a bold black line.

Figure A

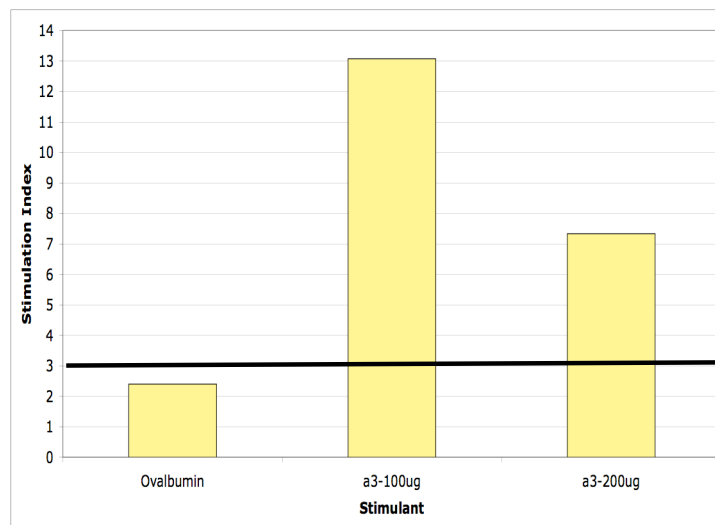


Figure B

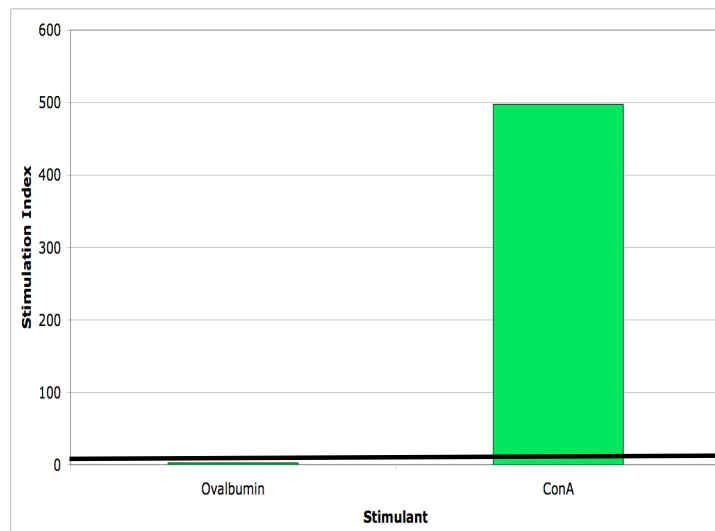


Figure 7.2.3. – Proliferative responses to recombinant human $\alpha 3$ in $\alpha 3$ KO mice.

Figures 7.2.3A-G show the calculated stimulation index for T cells from 7 $\alpha 3$ null mice after stimulation with the indicated peptide. As described previously, a stimulation index of 3 is marked with a bold, black line. Compared with data from the $\alpha 3$ WT mice, there is more variation in T cell proliferative responses and variation between $\alpha 3$ KO animals themselves.

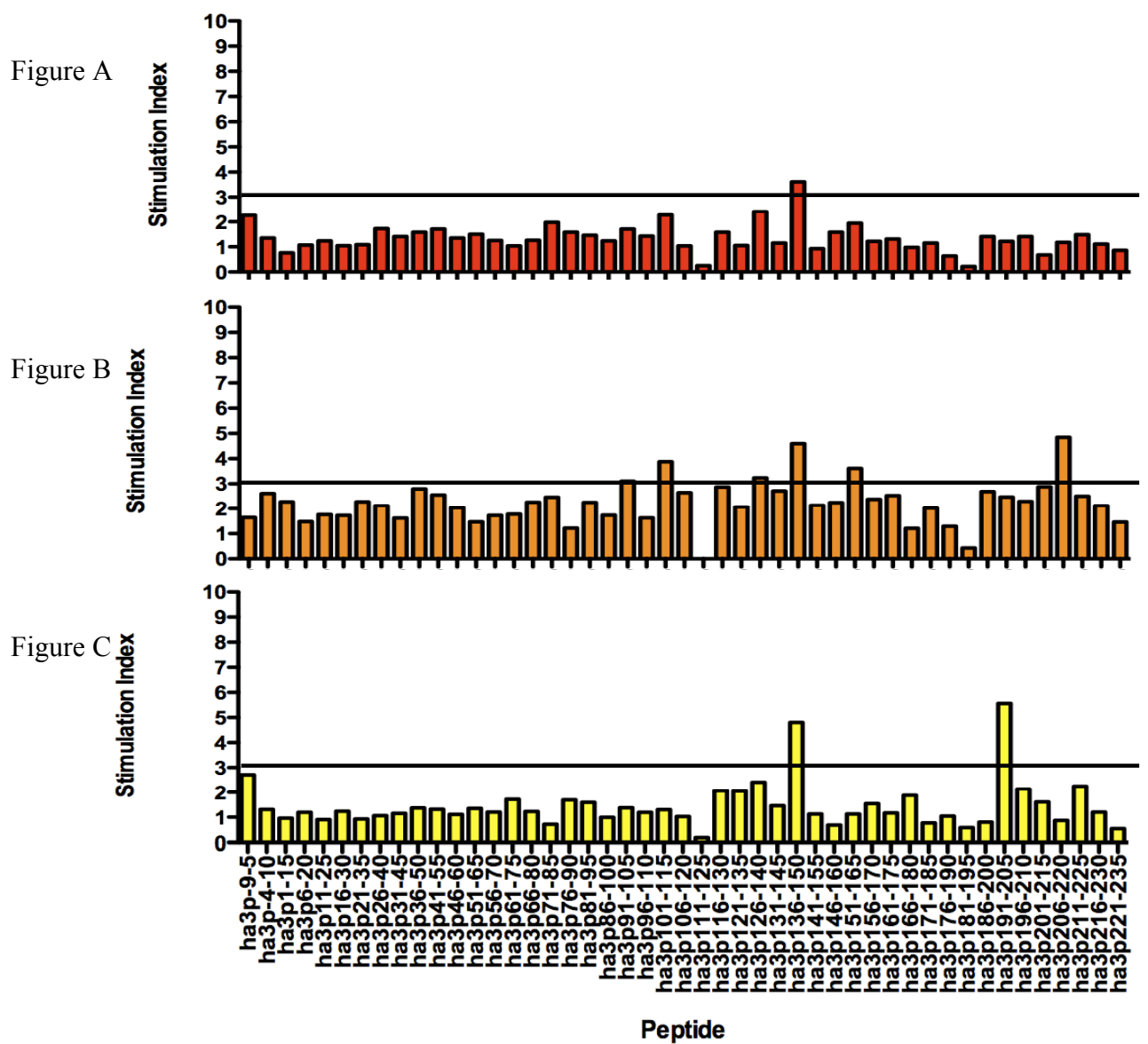


Figure 7.2.3. – Proliferative responses to recombinant human $\alpha 3(IV)NC1$ in $\alpha 3$ KO

DR15 mice

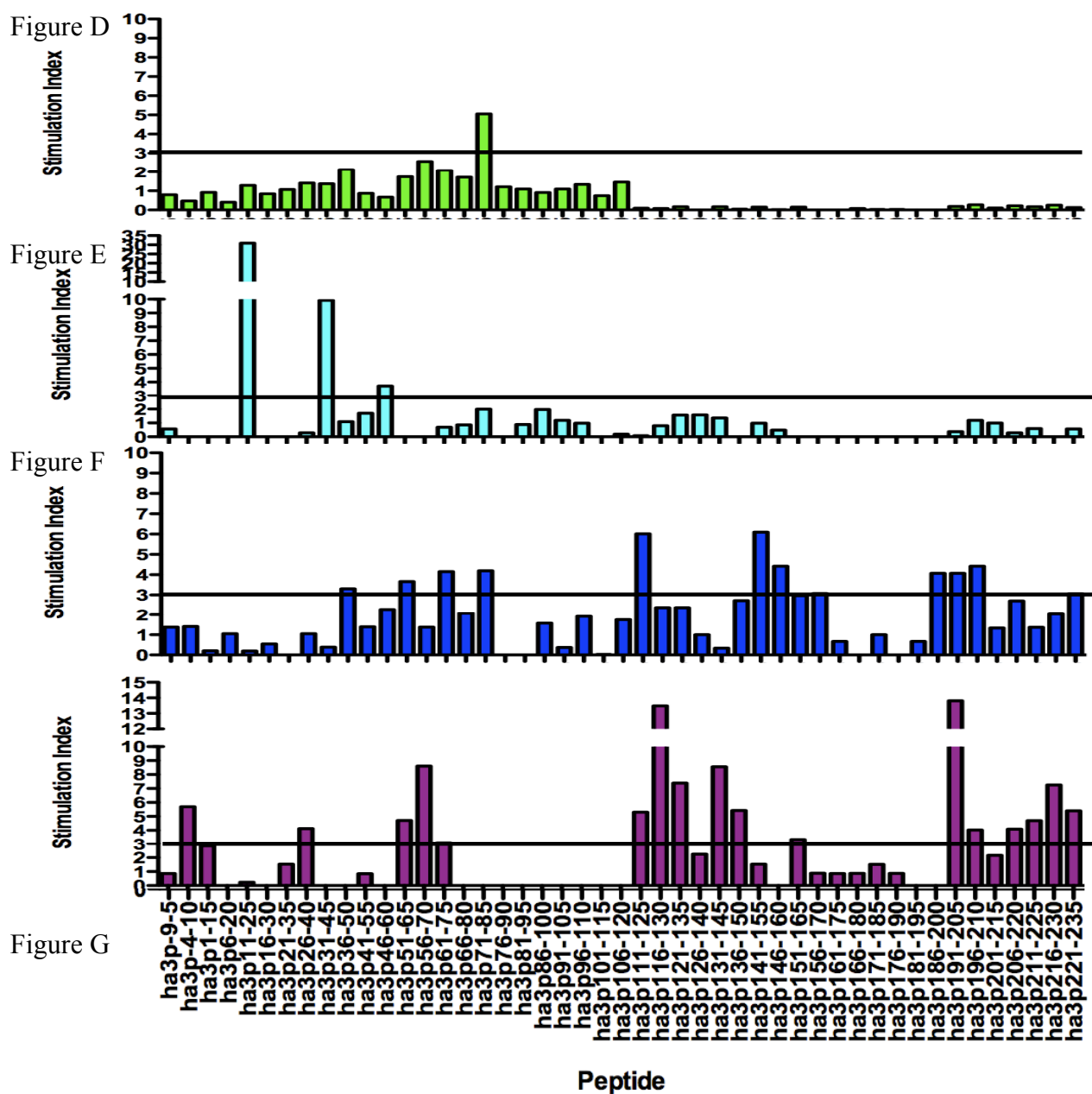


Figure 7.2.4. – Mean proliferative responses to $\alpha 3$ peptides by $\alpha 3$ KO mice

This chart combines all the data shown in Figure 7.2.3. (n=7) and combines it into a single chart. On the X axis is stimulating peptide, on the Y axis is mean stimulation index. A stimulation index of 3 is marked with a bold black line. From these data, four peptides give a mean stimulation index above 3, peptides ha3p11-25, 116-130, 136-150 and 191-205. These are marked with the number of animals out of the 7 tested that resulted in the stimulation index being above 3. As can be seen, peptide ha3p136-150 most consistently stimulated T cell proliferative responses (mean SI= 3.013, 5/7 mice).

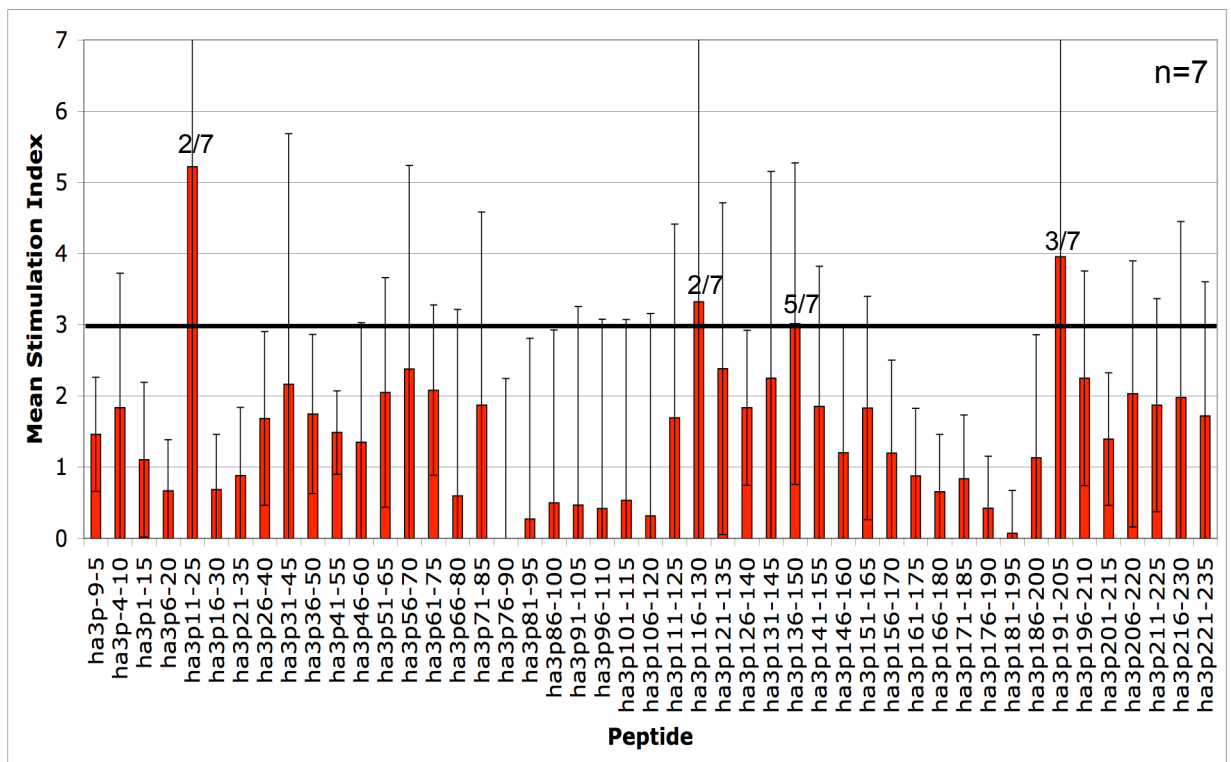
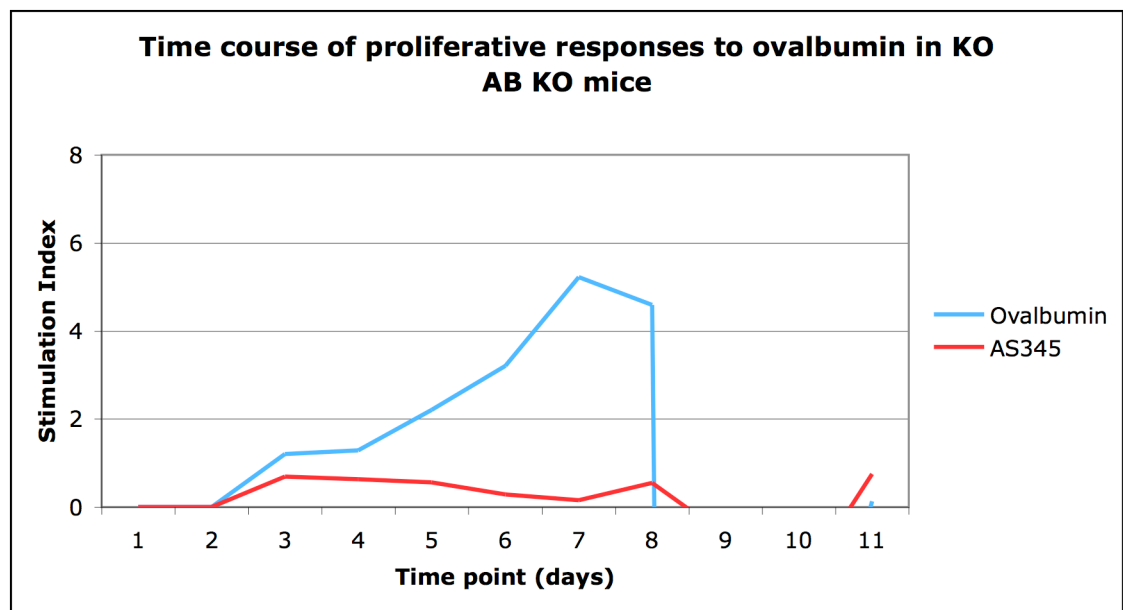


Figure 7.2.5. – Graph plotting time course of immune response to ovalbumin.

Lymphocytes from $\alpha 3$ null, HLA-DR15 positive mice immunised with ovalbumin were incubated with ovalbumin for the indicated time intervals shown before proliferation was assessed by tritium incorporation (X axis shows time until pulsing). An $\alpha 3$ peptide, AS345 or $\alpha 3(\text{IV})\text{NC1}$ S65-82 was used as a negative control over the same period. Tritium incorporation peaks at day 7 after in vitro stimulation with ovalbumin, while proliferative responses to the negative peptide remain low, suggesting little or no stimulation at any time point.



As was mentioned in Chapter 5, bacterial cells have different processing mechanisms for the production of protein, compared with human cells. The main difference being a lack of a crucial stage that enables the formation of disulphide bonds between specific cysteine residues and ensures a consistent conformation of a given cysteine-containing protein. This is particularly important in a molecule such as $\alpha 3$, when the NC1 domain amino acid sequence contains 12 cysteine residues. While all sequences could be folded into multiple conformations, the relatively high cysteine content of the $\alpha 3(\text{IV})\text{NC1}$ domain also increases the potential for various disulphide bond formation and therefore increased variability within the protein tertiary structure. Both bacterial recombinant $\alpha 3(\text{IV})\text{NC1}$ protein and human $\alpha 3(\text{IV})\text{NC1}$ extracted from tissue were used in experiments described within this Chapter. The experimental design is illustrated by Figure 7.2.1., and is the same as the experiments designed for the work described in Chapter 5.

In a similar experiment to those described in section 7.2.1, $\alpha 3$ null mice ($n=2$) were immunised with the $\alpha 3$ antigen preparation derived from human kidneys and purified by cation-exchange chromatography. The data obtained from mice immunised with human protein instead of recombinant protein also showed wide variation in the level of stimulation between peptides, and overall there was a stimulatory response akin to those seen following immunisation with recombinant $\alpha 3$. Only 1/2 mice (although the second showed an SI close to 3) showed a stimulatory response greater than 3 to the peptide ha3p136-150, but that number could increase had more mice been tested with the human $\alpha 3$ preparation. This was unfortunately not possible due to insufficient human protein.

Figure 7.2.6.– Proliferative responses of $\alpha 3$ KO mice to purified human $\alpha 3$ (IV)NC1.

These charts show the proliferative response data derived from mice immunised with human $\alpha 3$ (IV)NC1. The data is expressed as stimulation index (Y axis), with Figures A and B plotting the proliferative responses seen in T cells from two different mice, to the different peptides described on the X axis. As with all the data presented in form of a stimulation index, a threshold of 3 is marked with a bold black line.

Figure A

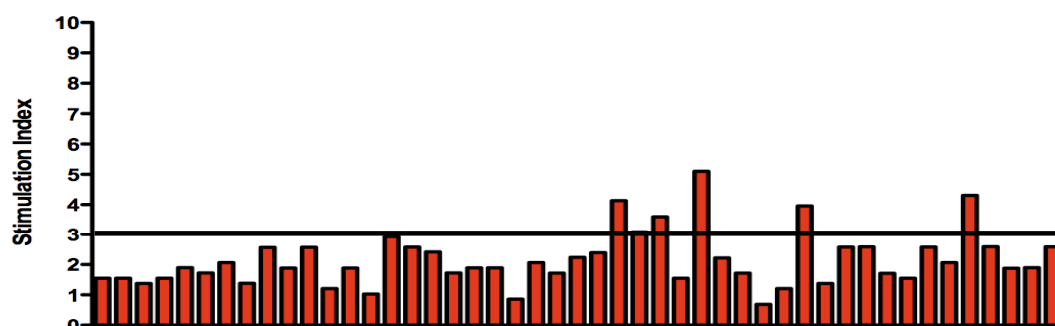
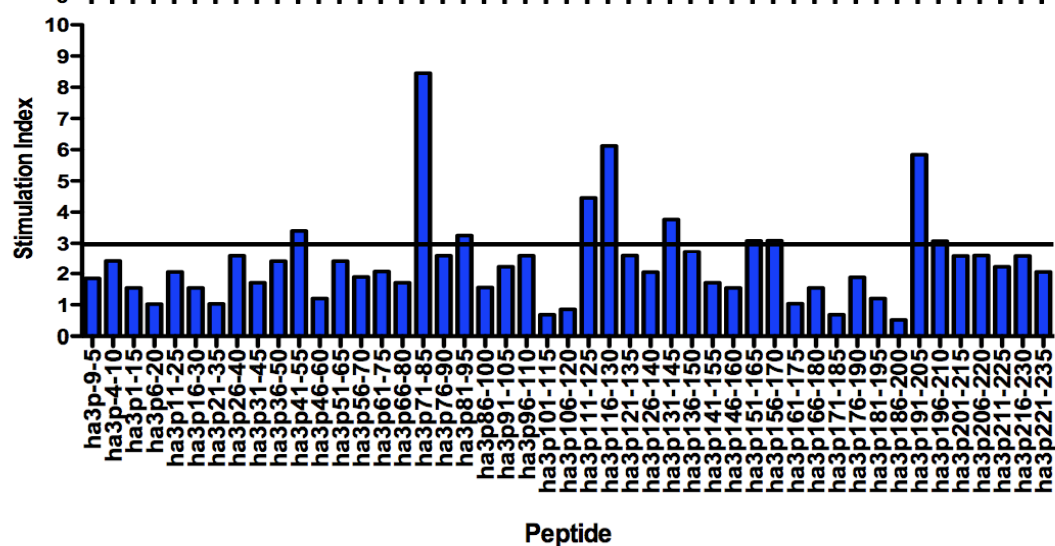


Figure B



The lowish levels of recall response to human $\alpha 3(\text{IV})\text{NCI}$ are unlikely to be as a consequence of too low a concentration of stimulatory peptides as the experiment employed the same concentration as used for the studies in $\alpha 3$ null mice immunised with $\text{rh}\alpha 3$. It may also be possible that the concentration of immunising antigen was insufficient to generate an immune response to all but the most stimulatory peptides. However, it can be seen that the $\alpha 3(\text{IV})\text{NCI}$ 136-150 peptide did stimulate a proliferative T cell response in one of the two mice immunised (Figure 7.2.6A).

The data obtained from immunising with human $\alpha 3$ is hard to interpret, mainly due to insufficient numbers to perform viable statistical comparisons. Repetition may have shown consistent patterns in responses, and at least would have allowed the significance of any consistency to be tested statistically. However, even in the 7 mice immunised with $\text{rh}\alpha 3$, it was difficult to isolate a specific trend. The peptide ha3p136-151 , caused a proliferative response in 5/7 mice tested, while the equivalent peptide in the mice immunised with the human $\alpha 3$ caused a proliferative response in 1/2 mice tested. This is likely to be a genuine finding, and the fact that there is a recall response to the ha3p136-150 peptide following immunisation with the human kidney-derived antigen suggests that despite the difference in conformation between the human protein and the recombinant protein, there are similarities in processing and presentation. It also suggests that there may be different ways to “unlock” the $\alpha 3(\text{IV})\text{NCI}$ protein that do not destroy the ha3p136-150 peptide. This could be through a different sequence of processing events, or through use of a different, possibly unidentified, processing enzyme.

The relative lack of consistent data may well be due to the fact that the murine line used in these experiments is not inbred, so variation is to be expected. It could be argued that in ensuring this murine line is not inbred, the variation in immune response to different peptides are more akin to the human population. However, when trying to isolate specific features of an antigen that could trigger autoimmunity or a lapse in tolerance, minimal variation renders data more easy to interpret.

7.2.4. – Responses by hybridomas

In order to make T cell hybridomas, isolated T cells (from a primed animal as for the recall response assay) are subjected to *in vitro* restimulation with antigen, followed by expansion with IL-2, before being fused to a myeloma line. This immortalises the cell and, if the fusion and serial dilutions are successful, results in clonal populations of T cell hybridomas with specific reactivity to a fragment of the antigen. This immortalisation enables multiple testing of the same clone using different antigenic fragments or antigen preparations and therefore offers a distinct advantage over primary cells. It is also easier to test these cells against a panel of peptides or possible stimulants. However, while hybridomas are good for specificity analysis, they do lose some of the qualities associated with primary immune cells. The hybridomas are specific for a very small fragment of a whole antigen. This reduces the conformational and processing constraints enforced by the APC as the T cell hybridomas will respond to a minimum concentration of the peptide presented by the APCs within the system. When T cell hybridomas are stimulated, they produce IL-2. This can be measured as concentration from 24 hour supernatants, by

ELISA. Another way of measuring the IL-2 produced would be by utilising cells that require IL-2 in order to survive, for example CTLL-2 cells (276). The supernatants can be used as feed for these cells and IL-2 concentration can be determined by proportional cell survival. However, these cells are notoriously difficult to grow in culture so it was decided to use the less sensitive ELISA instead.

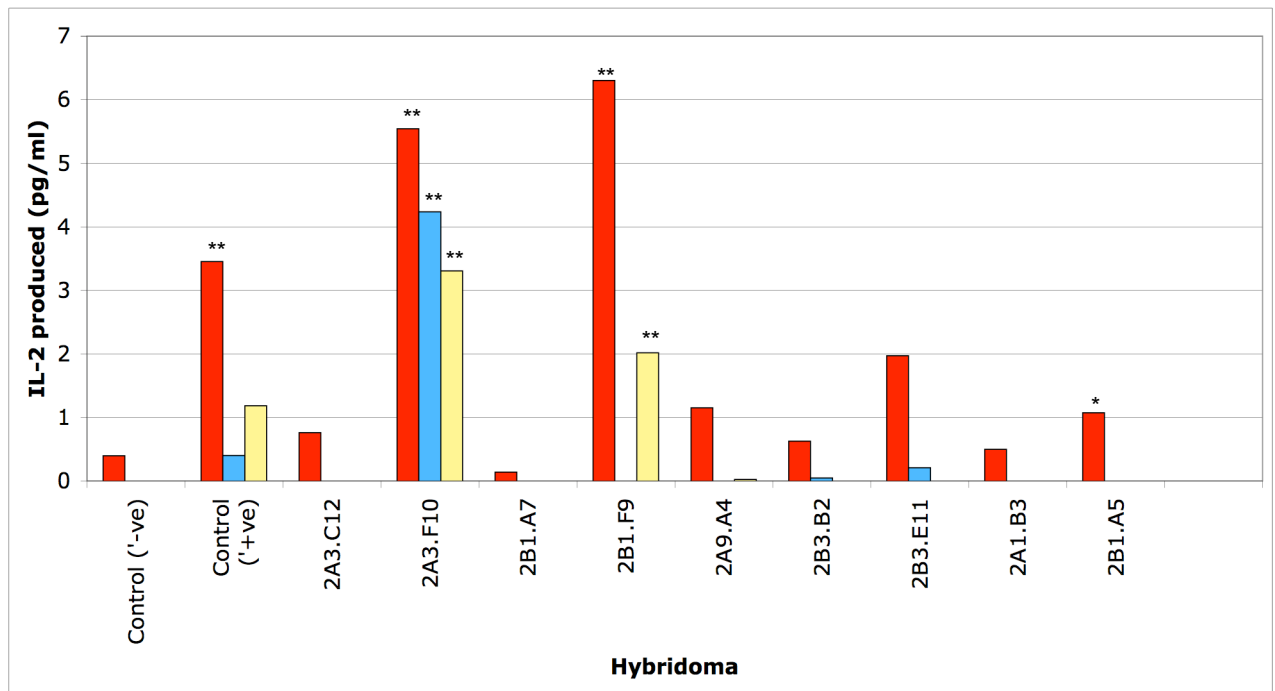
Overall, generation of hybridomas was attempted three times, using two mice on each occasion (six in total), but only one fusion reaction was successful. The first two attempts at making hybridomas produced no viable cells following fusion. The method to generate the hybridomas from DR15 A3v $\alpha 3$ KO mice is described in Chapter 2 (2.10.), but in brief, the mice were immunised as for the experiments described in sections 7.2.1 and 7.2.3 and as illustrated by Figure 7.2.1. The lymphoid cells obtained from each mouse were then restimulated with recombinant human $\alpha 3$ for 3 days, washed, then expanded for a further 3 days with a high concentration of IL-2/well (approximately four 24-well plates per mouse). These cells were then mixed at a 1:1 ratio with BW5146 cells and fused by disrupting the cell membranes with PEG. After washing to remove as much as possible of the PEG, the cells were gently resuspended in media and plated out undiluted, and at 1:3 and 1:9 dilutions in 96-well flat-bottomed plates. Each dilution was plated in duplicate and resulted in 6 plates per fusion reaction (effectively 6 plates per mouse). After 24 hours, selective media (containing HAT supplement) was added, and clones were selected and picked from 7-14 days. Cells were picked as showing clonal expansion if the expansion appeared to cluster in a specific region of the well and were picked when covering a maximum of 1/3 well surface area. The higher concentrations of cells (particularly the undiluted cell suspension post fusion) was

less likely to produce a fairly clean clonal population as a result of containing more cells initially. This in turn makes it more likely that more than one cell would undergo clonal expansion within the well, so a population of cells with responses to different regions of the $\alpha 3(\text{IV})\text{NC1}$ would be picked.

The hybridomas chosen to expand further and generate lines, were initially chosen for their survival in HAT then HT media, and then selected by a minimum of a 20% increase from background IL-2 production in response to $\alpha 3(\text{IV})\text{NC1}$. Using these criteria, 9 hybridomas were cultured (shown in Figure 7.2.7., * if $P < 0.05$, ** if $P < 0.01$, P is probability that amount of IL-2 produced above background concentrations was produced by chance, by unpaired T-test) and were shown to produce varying levels of IL-2 to whole $\alpha 3(\text{IV})\text{NC1}$. Of the clones selected, the highest consistent response to $\alpha 3(\text{IV})\text{NC1}$ was by hybridoma 2A3.F10, to both rh $\alpha 3$ (7.30pg, $P = 0.00001$) and hu $\alpha 3$ (4.99pg, $P = 0.000007$). Another hybridoma producing a significant amount of IL-2 in response to $\alpha 3$ was hybridoma 2B1.F9, although the responses from this hybridoma were solely to recombinant $\alpha 3$, suggesting that the responses were to a contaminating protein within that preparation. Since hybridoma 2A3.F10 responded to both human and recombinant $\alpha 3$, it is more likely that the responses seen by this hybridoma are to presented fragments of the $\alpha 3$ protein.

Figure 7.2.7. – Summary of IL-2 production by hybridomas.

This data was derived from HLA DR15 expressing $\alpha 3$ KO mice that had been immunised with rh $\alpha 3$ and the T cells restimulated and fused to myeloma cells to produce hybridomas that can produce IL-2 when stimulated. This chart shows each hybridoma line (plus negative clone as negative control and an older, well-established hybridoma as positive control). IL-2 concentrations produced in response to rh $\alpha 3$ are shown in red, to purified native hu $\alpha 3$ in blue, using a DR15-restricted B cell line as APCs. The final column in each set, shows IL-2 production in response to presentation of rh $\alpha 3$ by DR15-expressing, mitomycin c treated splenocytes (*= P< 0.05, **= P < 0.01, unpaired Student's T-test).



7.2.5. – Further characterisation of hybridoma responses

To determine which specific fragment of the $\alpha 3(\text{IV})\text{NC1}$ molecule the hybridomas responded to, each was set up against a panel of overlapping peptide sets. This was done to reduce the number of assays needed to test individual clones and to minimise reagent usage. The details of the sets are included in Appendix I, along with each peptide sequence and location (by amino acid position number) on the whole $\alpha 3(\text{IV})\text{NC1}$ domain. It was found that each hybridoma exhibited a distinct profile of responses and whilst most clearly responded to a single peptide set, only one hybridoma (2A3.F10, Figure 7.2.8E) showed responses to the major stimulating peptide seen in the initial lymphocyte stimulation assays (7.2.1. and 7.2.3.). This could be interpreted to mean that T cells responsive to $\alpha(\text{IV})\text{NC1}$ 136-150 do not survive the fusion process involved in making hybridomas, but this is pure conjecture. It is more likely to reflect the fact that very few hybridomas were produced from three different experiments (six mice).

Time did not permit the intended next step of measuring IL-2 responses to individual peptides from the stimulatory peptide mixtures, so to gain some indication of the likely T cell epitopes within the stimulatory peptide sets, the sequences were examined for DR15-binding motifs employing epitope-detection algorithms based on large bodies of experimental peptide binding data (258-260). Each MHC class II molecule, in this instance HLA-DR15, has an affinity for each peptide sequence that can be estimated. The data from each hybridoma and strongest stimulation set have been shown in table 7.2.9., with the likely stimulatory peptide with strongest

calculated binding for the DR15 molecule. This is calculated as an IC_{50} – that is the concentration of peptide predicted to displace 50% of labelled ligand from binding to, in this case, HLA DR15. This is the predicted value at which 50% of the DR15 molecules are inhibited (or occupied) by the peptide (nM). Where the hybridomas respond to more than one peptide set, it seems likely that these are not pure clonal populations of hybridomas so contamination most likely occurred at picking the original clone following fusion. This can be illustrated by comparing two of the charts within Figure 7.2.8. Both charts (as with all shown within the Figure) plot the IL-2 concentration produced in response to different stimuli. Figure 7.2.8F, a hybridoma line (2B1.A7) derived from the undiluted plates post-fusion shows a wider range of stimulation (sets 8, 14 and 15, 0.87pg/ml, 0.78pg/ml and 0.87pg/ml above background respectively, $P=0.017$, 0.033 and 0.036), compared with Figure 7.2.8A, a hybridoma line (2B3.B2) derived from the 1:3 dilution post-fusion that shows very specific stimulation by set 4 and the highest concentration of IL-2 produced (3.11pg/ml) in these experiments. However, this was not found to be statistically significant, due to variation within the triplicates.

Figure 7.2.8. – Charts showing IL-2 production of T cell hybridomas when incubated with sets of $\alpha 3(IV)NC1$ peptides.

IL-2 production measured by ELISA is shown in pg/ml for 8 hybridomas generated from $\alpha 3(IV)NC1$ immunised DR15-expressing, $\alpha 3(IV)NC1$ -KO mice. Statistically significant difference from background are marked * if $P < 0.05$, ** if $P < 0.01$ (unpaired Student's T-test, probability that IL-2 production in response to peptide set was by chance).

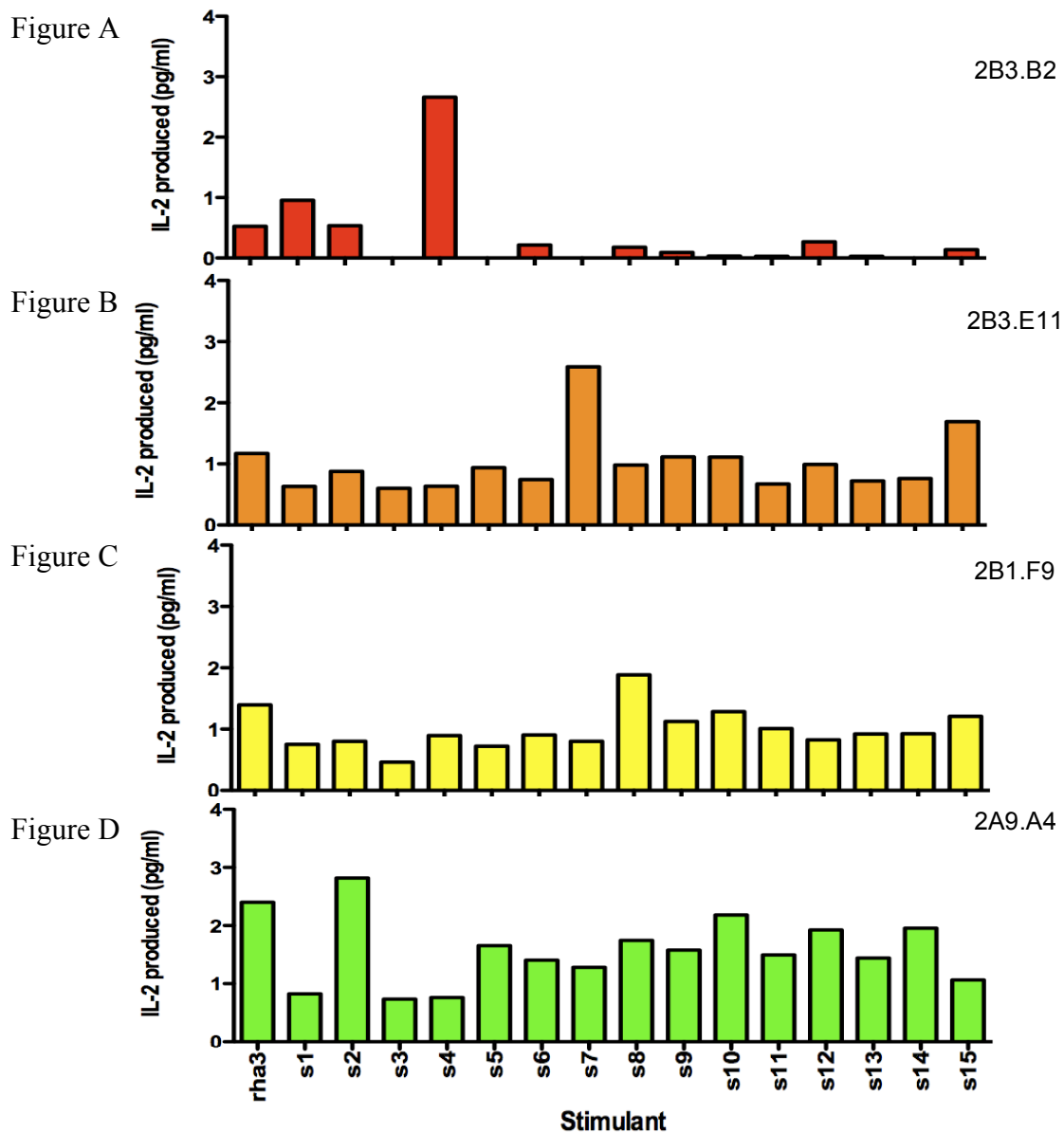


Figure 7.2.8. – Charts showing differential peptide responses from different hybridomas in response to overlapping $\alpha 3$ peptide sets.

As above, responses of hybridomas to overlapping peptide sets measured by IL-2 production. Significant differences from the background are marked * if $P < 0.05$ and ** if $P < 0.01$.

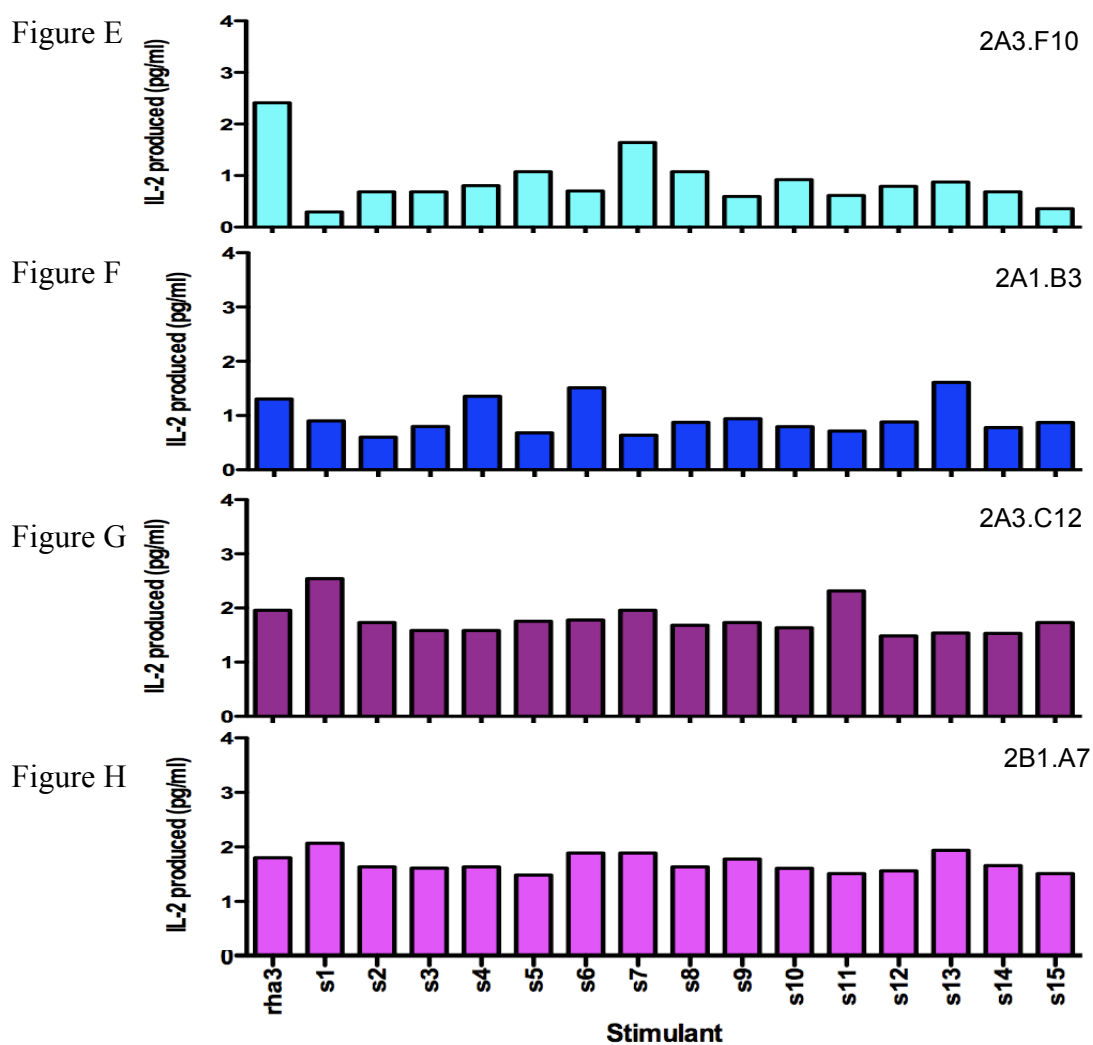


Table 7.2.1. – Table of hybridoma responses.

Table showing, for each hybridoma, the peptide sets eliciting the strongest IL-2 responses and likely epitopes within the constituent peptides deduced by determining to epitopes with greatest affinity for HLA-DR15 using the epitope prediction engine at:

http://tools.immuneepitope.org/tools/matrix/iedb_input?matrixClass=II

These numbers were obtained by pasting the peptide sequence into the website shown above and specifying which HLA-DR molecule was being studied. As can be seen here, the peptide with the lowest IC₅₀, so the lowest concentration required to inhibit 50% of the HLA-DR15 molecule, is α 3(IV)NC1 136-150. Despite that, only one of 9 hybridomas shows an increased IL-2 production in response to that set that includes it. Mean background concentration of IL-2 was 0.85pg/ml.

Hybridoma	Stimulatory Set	IL-2 produced (pg/ml)	Likely Peptide	Putative binding sequence	IC50 (nM)
2B3.E11	s7	2.71	α 3(IV)NC1 86-100	LMPMNMAPI	19.28
	s15	1.7	α 3(IV)NC1 206-220	PIPSTVKAG	725.55
2B3.B2	s4	3.11	α 3(IV)NC1 36-50	FVQGNQRAH	257.04
2B1.F9	s8	2.04	α 3(IV)NC1 96-110	MAPITGRAL	2687.99
2A9.A4	s2	2.95	α 3(IV)NC1 11-25	TRHSQTTAI	7629.66
	s10	2.2	α 3(IV)NC1 136-150	LWKGFSFIM	2.46
2A3.F10	s7	1.64	α 3(IV)NC1 86-100	LMPMNMAPI	19.28
2B1.A7	s6	1.58	α 3(IV)NC1 86-100	LMPMNMAPI	19.28
	s13	1.68	α 3(IV)NC1 171-185	ASPFLECHG	9163.28
2A3.C12	s1	2.41	α 3(IV)NC1 11-25	TRHSQTTAI	7629.66
	s11	2.62	α 3(IV)NC1 146-160	SFIMFTSAG	93.51
2A1.B3	s1	2.067	α 3(IV)NC1 11-25	TRHSQTTAI	7629.66

Clonality is more likely when the fusion products were plated at higher dilutions, but the single peak of IL-2 production in response to one specific peptide set is not always seen in the clones derived from these plates. In particular, hybridoma 2A9.A4 (Figure 7.2.6A) was derived from the 1:9 dilution post-fusion and is seen to have four statistically significant peaks of IL-2 production (set 6, set 8, set 12 and set 14). This suggests either more than one clone within the original well was picked and expanded, or that there is some degree of similarity between peptides within those two sets (sets 6, 8, 12 and 14).

The hybridoma 2A9.A4 also shows stimulation to sets 2 and 10, but this was not found to be statistically significant, again probably due to variation between the triplicate samples. However, the sequences within these sets shows 60% the same sequence and was shown to be 73% homologous when aligned (by BLAST 2), suggesting that cell responsive to one peptide sequence within set 2 could also be responsive to peptides within set 10. Of additional interest is the knowledge that set 10 contains the Goodpasture antigen peptide $\alpha 3(\text{IV})\text{NC1}$ 136-150, the peptide to which all Goodpasture patients generate an immune response, and the immunodominant peptide within the Goodpasture antigen.

Figure 7.2.8. also emphasises the point made above about clonality. This is particularly well illustrated by comparing Figures 7.2.6A, 7.2.6E and 7.2.6G. The hybridoma used in Figure 7.2.6C was grown from a single well in the 1 in 3 dilution plate and clearly shows a single peak of IL-2 production in response to peptide set, s4. By way of contrast, the hybridomas illustrated in Figures 7.2.6F and 7.2.6H were both derived from the undiluted fusion mixture, and 7.2.6H shows no real peak of

response, more a generic production of IL-2, while 7.2.6F shows a few peaks of IL-2 production, suggesting a lack of clonality.

Student's T-tests for these data found that, although the means of the data were significant by eye, these were not always statistically significant. There was some variation between samples (each was carried out in triplicate), so many of the peaks that appear significant by eye are not statistically significant by Student's T-test. Other peaks that appeared to be similar to the background were classed as statistically significant by an unpaired Student's T-test. While there was variation between triplicates, and those producing the greatest concentrations of IL-2 showed the greatest variation between samples, the statistics are such that increased variation detracts from the data. For this data set, some mean values that are high have not been calculated as significant due to variation within the triplicates used to calculate the mean. The T-test assumes that all data points are equally valid, so if there is wide variation within a sample, the sample itself must have wide variation. This is unlikely to be the case for the data presented here, but time constraints meant that repetition was not possible. This data should therefore be treated with caution. It should not be discarded, but the peaks that were significant by eye but not statistics should not be treated as significant until further study regarding the IL-2 productive response of the hybridomas has been undertaken, and the data presented here, corroborated.

7.2.7. – Histology from immunised $\alpha 3$ null mice

In keeping with experiments on $\alpha 3$ wild-type mice, kidneys were harvested from immunised mice and unimmunised control animals, fixed in 10% neutral formalin and paraffin wax embedded. Sections were then cut for haematoxylin and eosin staining, but, despite the differences in peptide responses compared to wild type animals, there were no morphological differences either between immunised and unimmunised $\alpha 3$ KO mouse kidneys (Figure 7.2.10.), or compared to wild type sections (Figure 6.2.7). This is not entirely surprising – alterations in kidney morphology are notoriously hard to induce. The experimental procedure followed is probably too short-term and more mild a schedule than would be required for distinct morphological differences to be induced and observed.

7.3. – Discussion

The variation seen between the two sets of data was somewhat surprising. In particular, the variation in stimulatory peptides seen in the hybridomas, compared with the lack of variation within the lymphocyte stimulation assays. However, the two sets of experiments, while suitable for validation, do determine different things. The lymphocyte stimulation assay assesses the immunodominant peptide and reflects collective immune response. Hybridomas, by comparison, are derived from a single cell. In order to use them to assess an immune response, hundreds of hybridomas from a single animal, reacting to the entire of an antigen are required. This enables some comparisons to be made about the overall reactivity of each peptide in comparison to each other. The original intention behind this work was to generate

Figure 7.2.9. – Photographs of haematoxylin and eosin stained kidney sections from immunised and unimmunised mice.

These photos show unimmunised mouse kidney (Figure A), ovalbumin immunised mouse kidneys (Figure B), rh α 3 immunised mouse kidney (Figure C) and hu α 3 immunised mouse kidney (Figure D). The images were captured at x400 magnification. As is shown, all glomeruli look normal irrespective of immunisation schedule.

Figure A – Naïve mouse

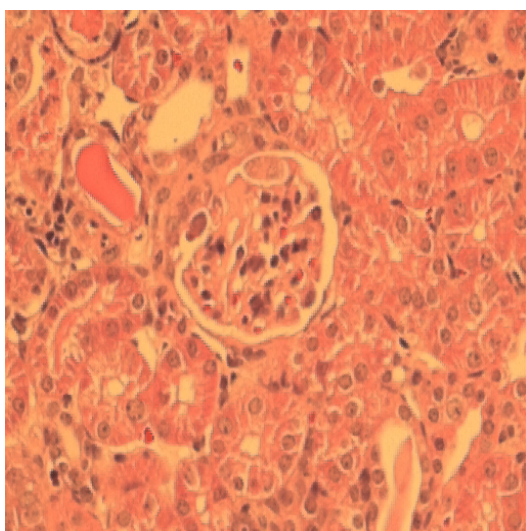


Figure B - Ovalbumin

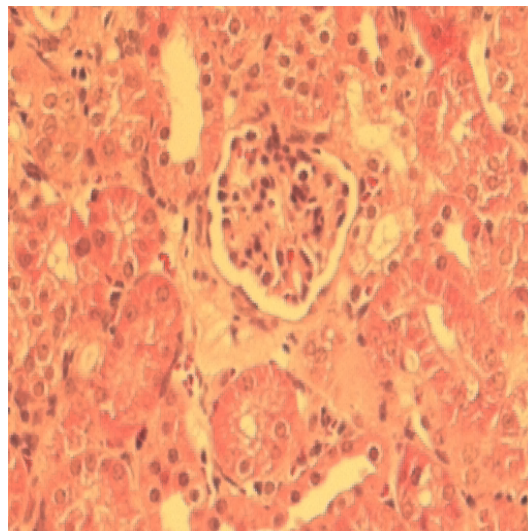


Figure C - rh α 3

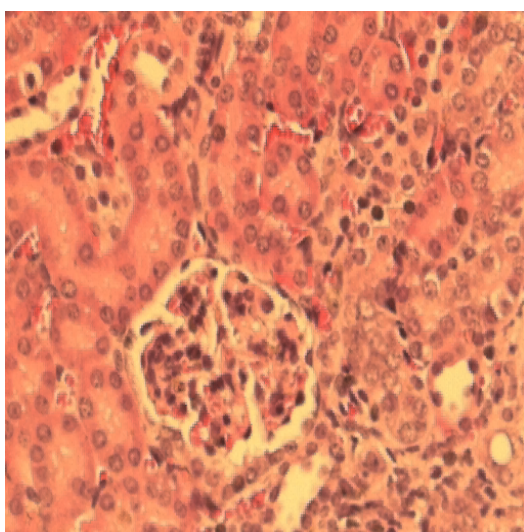
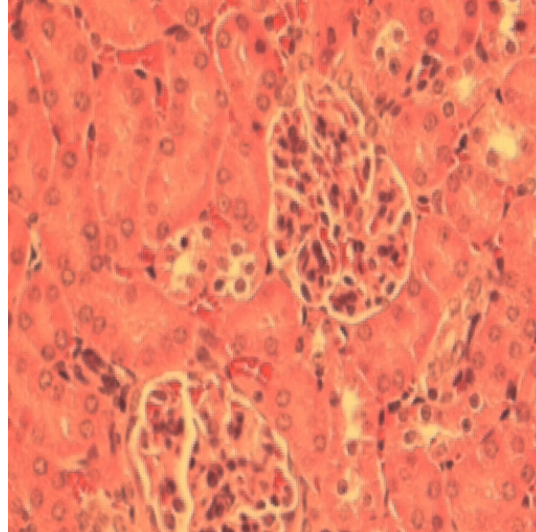


Figure D - hu α 3



lots of hybridomas and corroborate the recall response data. With the numbers of hybridomas actually generated in this work, this corroboration was not possible. While it would be expected that proportionally more hybridomas would respond to the immunodominant peptide, it remains possible that other T cells with affinity for less dominant peptides can be fused, selected and cultured prior to testing. In effect, the production of hybridomas ensures that a single cell can be tested multiple times with different peptides to determine single cell peptide binding specificity. The lack of hybridomas that respond to the immunodominant peptide is more likely to be a result of the few hybridomas successfully cultured, rather than an accurate reflection of the overall immune response in the animal from which the original T cells were obtained.

These experiments do illustrate how variable the immune responses to exogenous antigen can be in relatively outbred mice and as in man. On the one hand, $\alpha 3$ null mice immunised with either rh $\alpha 3$ or hu $\alpha 3$ consistently generate the greatest immune responses to the Goodpasture $\alpha 3(\text{IV})\text{NC1}$ fragment, $\alpha 3(\text{IV})\text{NC1}$ 136-150. On the other hand, cells from $\alpha 3$ null mice, immunised with the same rh $\alpha 3$ preparation, then fused to myeloma cells in order to make hybridomas result in immune responses to a variety of peptide sets, but only 1 out of 8 hybridomas did respond to the set that contained the Goodpasture antigen. This is probably due to the reasons described above.

The consistent response to $\alpha 3(\text{IV})\text{NC1}$ 136-150 in the lymphocyte stimulation assays presents with a conundrum. The peptide fragment $\alpha 3(\text{IV})\text{NC1}$ 136-150 that stimulated T cells from Goodpasture patients and HLA-matched controls (1), as well as the work described within this Chapter, encompasses a cleavage site for cathepsin

D, and in particular is the site of very early processing events for the whole $\alpha 3$ NC1 domain (71). This peptide should therefore be destroyed during early processing events and cleavage at this site is also known to be a key stage in “unlocking” and processing the rest of the molecule. A possible explanation for this could be incomplete cleavage, in which the protein is cleaved enough to “unlock” the protein, but insufficiently to destroy the peptide. It is also possible that this peptide fragment is presented as a result of aberrant processing through either faulty enzyme or faulty $\alpha 3(\text{IV})\text{NC1}$ sequence. The latter seems unlikely as the genetic sequence of the $\alpha 3$ chain in patients with Goodpasture’s disease has been sequenced and no mutations have been found (277). However, all published data from my supervisor’s laboratory regarding processing and presentation of $\alpha(\text{IV})\text{NC1}$ has used recombinant bacterial $\alpha 3(\text{IV})\text{NC1}$ which has variable conformations within batches, and may well show different processing as a result of differences in accessibility for processing enzymes. The fact that data obtained using $\alpha 3(\text{IV})\text{NC1}$ derived from human kidneys also resulted in recall responses to some of the peptides causing recall responses from mice immunised with recombinant $\alpha 3(\text{IV})\text{NC1}$ suggests that this is a real finding. The faulty enzyme hypothesis seems unlikely as the work described in this chapter suggests processing is similar in mouse as in man and the mice used are not lacking cathepsins D or E, which would be the more obvious candidates as defective enzymes (71). Another possible mechanism enabling presentation of a previously cleaved fragment is protein splicing, in which protein fragments are spliced together after processing in order to generate an immune response (278). It has since been shown that even peptide fragments spliced incorrectly can enable an immune response to be generated following presentation on the APC surface (279). This

remains a possibility for Goodpasture's disease and in this transgenic mouse experiments, but far more work would need to be done including time-consuming and expensive protein tracking experiments to determine where the protein is being cleaved and subsequently, where it is then being spliced back together. This also generates a question whether the peptides to produce the $\alpha 3(\text{IV})\text{NC1}$ 136-150 fragment are being spliced back together deliberately, or whether it is purely due to the proximity of peptides to each other within the endosome.

The work described within this chapter investigating immune responses to the $\alpha 3(\text{IV})\text{NC1}$ protein in $\alpha 3$ null mice found a surprising variation in the level of immune response both to recombinant human $\alpha 3(\text{IV})\text{NC1}$ and to human $\alpha 3(\text{IV})\text{NC1}$ isolated from tissue. Using the recombinant protein, the most consistent immune response was generated to the Goodpasture fragment, $\alpha 3(\text{IV})\text{NC1}$ 136-150. However, since these mice do not express the $\alpha 3(\text{IV})$ protein, it was expected that more areas of the protein would result in T cell proliferation, since it is a foreign antigen to these animals. Overall, these animals had lower than expected immune responses. Even the whole recombinant $\alpha 3(\text{IV})\text{NC1}$ at various concentrations only generated limited immune responses, but this could be because the protein was in highly concentrated (8M) urea so addition of large volumes of the rh $\alpha 3$ would not be particularly conducive to cell growth (maximum final concentration of urea in any experiment was 0.376M), this was tested and has been shown in Chapter 6 (Figure 6.2.5). This high concentration of urea was used to keep reasonably high concentrations of recombinant $\alpha 3$ in solution and subsequent dilution caused the protein to precipitate. It is possible, although unlikely, that as $\alpha 3(\text{IV})\text{NC1}$ naturally forms a trimer with $\alpha 4$ and $\alpha 5(\text{IV})\text{NC1}$ domains, the precipitation was a direct result

of the protein aggregating to form a more stable di- or trimer and to protect otherwise exposed hydrophobic domains, in the more dilute urea solutions.

The low level of *in vitro* immune response to peptides cannot be for the same reason, as the peptides are all chemically synthesised. This could be due to two possibilities: firstly, that there is genuinely limited immune responses to peptides other than $\alpha 3(\text{IV})\text{NC1}$ 136-150; or secondly that the cells used during the experiment were not a healthy population, resulting in high levels of cell death and therefore limiting the immune responses seen following harvesting. The second reason is possible, as the lymph nodes isolated were visibly in close proximity to adjuvant used during the immunisations. While the cells were washed well prior to plating, the adjuvant used to generate an immune response (in conjunction with the recombinant $\alpha 3(\text{IV})$ protein) is fairly toxic and promotes T cell activation. The adjuvant seen could have been near the lymph node for up to two weeks, with the potential to affect the health of cells within that area. These concerns are also applicable to the data obtained from DR15-expressing $\alpha 3$ WT mice and presented in Chapter 6, although a lack of immune response in these mice does support robust tolerance.

T cells from $\alpha 3$ immunised DR15 $\alpha 3$ WT mice do show *in vitro* immune responses to whole bacterial recombinant $\alpha 3(\text{IV})\text{NC1}$, but not to individual peptides, suggesting that the rh $\alpha 3$ contains some additional contaminant and that tolerance to the $\alpha 3(\text{IV})\text{NC1}$ protein is robust. It was expected that more immune responses to the $\alpha 3(\text{IV})\text{NC1}$ peptides would be seen in T cell from the DR15 $\alpha 3$ null mice, as the protein is foreign so would generate an immune response as seen to other exogenous proteins (ovalbumin, Figure 7.2.5.).

While this is true to some extent, the immune responses seen were at a lower level than was expected and suggesting that the antigen preparation used to immunise the mice was less concentrated than expected. It also suggests that the protein itself may not be that immunogenic, a suggestion that may also partially explain the scarcity of disease in humans. However, this would also infer that foreign proteins are subjected to different levels of immune response and this is not the case.

It could be argued that the cells made into hybridomas have been through a lot more processing and manipulation than those obtained relatively simply by disaggregating lymph nodes and being kept in media containing a known concentration of $\alpha 3(\text{IV})\text{NC1}$ peptide. This, however, should not have altered the inherent ability of the T cell to recognise a specific fragment of antigen in conjunction with the presenting MHC class II molecule. In particular, individual variation may well play a role, especially as all the hybridomas that were made successfully were derived from one mouse. Two $\alpha 3$ null mice were immunised and used in the process, but all the hybridomas found to be specific for the $\alpha 3(\text{IV})\text{NC1}$ molecule were from the second mouse (hence “2” at the front of the hybridoma nomenclature). This automatically means that all the hybridomas are obtained from a limited immune repertoire and since the same mouse could not be tested simultaneously for peptide recall responses, it is not known whether this mouse just generally showed reduced recognition of the $\alpha 3(\text{IV})\text{NC1}$ 136-150 peptide fragment, or whether any cells that did recognise this fragment did not survive the fusion process. The latter reason is unlikely because other T cells were perfectly able to survive the fusion and mount a cellular response to the relevant peptide fragment. In

addition, there are already hybridomas that do recognise this fragment within our laboratory, having undergone exactly the same fusion protocol.

Part of the aim of this chapter was to try to produce individual hybridomas that respond to different fragments of the $\alpha 3(\text{IV})\text{NC1}$ molecule, and effectively producing a panel of hybridomas for *in vitro* immunological studies. While this was well within the scope of my project, the practicality of actually being able to do so rendered this a very difficult aim to achieve. To have established 8 new hybridoma lines with different peptide specificity is a substantial effort, but future work could definitely include taking this further and developing additional hybridoma lines with different specificities from those developed here. As a panel, the 8 clones successfully cultured here, plus some clones to different peptide sequences previously developed within the lab, and with the future development of clones with fine specificities for the remaining peptides within the $\alpha 3(\text{IV})\text{NC1}$ protein, could be used to determine T cell responses. These could be to study responses to specific fragments and modified fragments to determine essential amino acid residues for recognition, binding and presentation by the MHC class II HLA-DR15 molecule. They would also allow more specific study of processing mechanisms and criteria.

The work described within this chapter shows consistent immune responses by primary T cells derived from $\text{rh}\alpha 3$ immunised DR15-expressing $\alpha 3\text{-KO}$ mice to the $\alpha 3(\text{IV})\text{NC1}$ 136-150 peptide. This is the peptide to which T cells from 100% Goodpasture patients also respond. This suggests that the $\alpha 3(\text{IV})\text{NC1}$ 136-150 peptide is the immunodominant peptide, despite processing requirements that would suggest it should not be presented on the APC surface at all (71). Despite the similarities between the results, the human and mouse responses cannot be directly

compared. Firstly, the *in vitro* responses discussed within this chapter are obtained from a humanised transgenic mouse with limited MHC class II repertoire, while the human data published, although based on Goodpasture patients, has no such limitation, despite all the patients described expressing HLA-DR15. In addition, Goodpasture patients have tolerance mechanisms that do not prevent autoimmune responses, while the data presented within this chapter describes murine immune responses to exogenous antigen since these mice do not express $\alpha 3(\text{IV})\text{NC1}$. However, this work shows that responses in DR15 positive, $\alpha 3(\text{IV})$ null mice, in the absence of tolerance, are similar to those seen in patients with Goodpasture's disease. This implies that the mice used in Chapter 6, HLA DR15-expressing $\alpha 3$ WT, could be a useful tool for studying the mechanisms causing Goodpasture's disease in more depth. This is on the proviso that tolerance mechanisms in these mice can be broken (280). Conversely, the mice used in this chapter, HLA DR15-expressing $\alpha 3$ -KO mice, could be used to study induction of tolerance and whether the induced tolerance to the $\alpha 3(\text{IV})\text{NC1}$ domain could subsequently be broken (281).

In summary, the work described in this chapter suggests that the $\alpha 3(\text{IV})\text{NC1}$ 136-150 is the immunodominant peptide against which immune responses are most likely to be directed, in the DR15-expressing $\alpha 3$ -KO mouse developed in Chapter 4. The work undertaken to develop hybridomas suggests that this peptide is not the sole immunologically responsive peptide, but that other peptides may need to be presented at much higher concentrations on APC surfaces in order to generate a similar immune response. The work described in Chapter 3, in developing a peptide adjuvant to improve specific uptake into B cells is one way in which to potentially exploit this mechanism.

Chapter 8 – Final Conclusions and Future Directions

This project investigated epitope selection in the presence and absence of tolerance and the subsequent immune responses. While the majority of the work concentrated on generating antigen and testing the CD4 T cell responses, there was some additional work investigating the possibility of exploiting mechanisms of antigen uptake.

Early work concentrated on improving specific B cell-mediated uptake of peptides by developing a B cell-specific fusion partner, protein L, could be explored further. This protein shows promise for use as a fusion partner as data is shown illustrating the protein L binding and being internalised by B cells (Chapter 3). In order to study the effect of processing on presentation, there are two influencing factors that should be considered, namely uptake and presentation. If uptake can be made consistent, specific processing preferences are more easily identified. Protein L has the potential to be used to augment uptake such that uptake is no longer a limiting factor in determining protein processing and presentation, enabling identification of specific immunological peptide sequences presented on the APC surface. Future work could therefore include linking protein L to a peptide of interest and testing T cell responses using the pulsed B cells as APCs and using peptide-specific T cell hybridomas as a read out (IL-2 production). The whole Goodpasture antigen could also be linked to protein L and resultant T cell immune responses tested from a panel of T cell hybridomas generated against overlapping

regions of the Goodpasture antigen (work started in Chapter 7 with the generation of 8 hybridoma lines reactive to different fragments of the Goodpasture antigen). This could enable further investigation of the processing mechanisms involved in processing and presentation of the Goodpasture antigen.

The protein L studies were undertaken in order to produce a protein capable of specifically binding B cells and with potential for use as a fusion protein to improve uptake of specific antigen into the B cells for processing and presentation. This protein was the bacterial protein L (as described in Chapter 3) and was shown to be capable of binding and being taken up into B cells, although further work to link the protein L to specific antigen was limited due to time constraints.

Additional early work concentrated on generating an *in vivo* system within which it would be possible to test for immune responses to a specific antigen. Two transgenic mouse lines were crossed to produce a new transgenic line that did not express mouse class II molecules, that expressed the HLA-DR15 molecule, and could be $\alpha 3(\text{IV})\text{NC1}$ positive, heterozygous or null, enabling exploration of $\alpha 3$ -specific immune responses in the presence and absence of tolerance (Chapter 4). The transgenic mouse line generated showed promise for use investigating mechanisms underlying autoimmunity, particularly Goodpasture's disease, but needed care to maintain. There was a distinct female: male skew (3:1), certain genotypes never occurred in males, and some female genotypes were unsuitable for use in breeding.

In addition to the generation of the transgenic mouse line, early work also concentrated on generating sufficient bacterial recombinant human antigen ($\alpha 3(\text{IV})\text{NC1}$) for the immunisation experiments as well as isolating native human

$\alpha 3(\text{IV})\text{NC1}$ from human kidneys. Later in the project, work was undertaken to produce a vector for the transfection of human embryonic kidney cells such that successfully transfected cells would produce a soluble form of $\alpha 3(\text{IV})\text{NC1}$ (Chapter 5).

Chapter 6 presents the work carried out using the mouse class II KO, DR15-expressing, $\alpha 3$ wild-type mice to determine the immune responses to $\alpha 3(\text{IV})\text{NC1}$ and $\alpha 3(\text{IV})\text{NC1}$ -derived peptides. These mice showed no responses to fragments of $\alpha 3(\text{IV})\text{NC1}$ despite a moderately severe immunisation schedule. These data emphasise the need for exceptional circumstances are necessary in order for the immune system to respond to self-antigen. The fact that these experiments were unable to find any response to $\alpha 3$ suggests that tolerance is robust. However, recent work by Zou *et al.* has shown that healthy humans do have circulating Goodpasture antigen reactive T cells (3). They hypothesised that these autoreactive T cells escaped tolerance mechanisms as a result of reacting to a peptide fragment that should be destroyed by lysosomal processing. No T cells specific for the rapidly-destroyed peptides could be demonstrated in the $\alpha 3(\text{IV})\text{NC1}$ -expressing transgenic mice, even after immunisation with human $\alpha 3(\text{IV})\text{NC1}$, but this observation does not greatly undermine the hypothesis as no peptide specific responses were detected whatsoever. Additionally, these studies were undertaken using human $\alpha 3(\text{IV})\text{NC1}$ and human peptide sequences in a transgenic mouse model, so there may be some species-specific differences. It would have been interesting to explore other means of breaking tolerance to $\alpha 3(\text{IV})\text{NC1}$ in these mice, for example by removing immunosuppressive hormones, immunising with antigen and administering additional APCs or by antibody blockade of the CTLA-4 receptor (282).

The mice that did not express $\alpha 3$ (mouse class II KO, DR15-expressing, $\alpha 3$ KO) did produce an immune response to $\alpha 3(\text{IV})\text{NC1}$ and specifically to $\alpha 3(\text{IV})\text{NC1}$ 136-150 – a region of the protein similar to the immunodominant peptide implicated in the development of Goodpasture’s disease in humans ($\alpha 3(\text{IV})\text{NC1}$ 131-150). Hybridomas generated using mice with this genotype had specificities to various peptides within the $\alpha 3(\text{IV})\text{NC1}$ domain, including $\alpha 3(\text{IV})\text{NC1}$ 136-150. It was not possible to fully characterise all the hybridomas made, but they have the potential to be useful reagents to probe specific processing mechanisms (Chapter 7).

Overall, the data presented within this thesis is surprising compared with published data regarding immune responses in Goodpasture patients (1). It shows that, despite the requirement for the $\alpha 3(\text{IV})\text{NC1}$ protein to be “unlocked” by enzymatic cleavage in a specific sequence of processing events (71), the initial cleavage site is also the peptide sequence to which there is overlap between major immune responses seen in man (1) ($\alpha 3(\text{IV})\text{NC1}$ 131-150) and in $\alpha 3$ KO mice ($\alpha 3(\text{IV})\text{NC1}$ 136-150, Chapter 7). In the mice more similar to man, the $\alpha 3(\text{IV})\text{NC1}$ WT mice, there were no immune responses to any human peptide following immunisation with human $\alpha 3(\text{IV})\text{NC1}$. This raises questions for further examination and investigation. Possibilities include investigating the nature of the immunogenic peptide, reasons for it being presented and possible mechanisms by which it is preserved for presentation by the immune system. They also include further investigating possible mechanisms causing the autoimmunity, mechanisms for breaking or avoiding tolerance, and whether tolerance can be reinstated in autoimmune disease.

As far as processing of the $\alpha 3(\text{IV})\text{NC1}$ molecule is understood, like the tetanus toxin (68, 69), $\alpha 3(\text{IV})\text{NC1}$ requires a specific cleavage event to occur before the remainder of the protein, and particular regions containing T cell epitopes, can be processed. It has been shown that this cleavage event effectively “unlocks” the remainder of the molecule (71). The peptide encoding that region ($\alpha 3(\text{IV})\text{NC1}$ 131-150 and $\alpha 3(\text{IV})\text{NC1}$ 136-150, in man and mouse respectively) has also been shown to be the most immunogenic in both humans (Goodpasture patients (1), healthy individuals (3)) and $\alpha 3$ KO mice (Chapter 7). Since this peptide should be destroyed during processing events, the protein could be incompletely processed enabling presentation, or the peptide could be spliced back together during post-processing and MHC class II binding events. The first hypothesis could be tested by generating point mutations within the peptides and determining which residues are essential for binding and generating an immune response, and determining which mutations render the peptide unable to be processed, so which residues are essential for processing, provided T cells recognise the APC. The combined data could help determine essential residues for both binding and processing and also determine whether there is overlap between the two.

The second hypothesis of peptide splicing would be more difficult to examine. Published work by Vigneron *et al.* in 2004 and Warren *et al.* in 2006 (278, 279) showed evidence of peptide splicing in immune responses from CD8-positive T cells. They showed that specific peptides could be digested within the proteasome and spliced together such that a reactive peptide was presented, within MHC class I molecules, to the clonal CD8 T cell line isolated from a sample. They subsequently showed, by high performance liquid chromatography (HPLC) and mass spectrometry

(MS), that the proteasome was essential for the splicing reaction and that the cleavage events preceding splicing were essential for generating sufficient energy for the splicing reaction to occur. The processing mechanisms are different for MHC class II molecules but the hypothesis could be tested by using the reactive peptide of the Goodpasture antigen ($\alpha 3(\text{IV})\text{NC1}$ 131-150) added to DR15-expressing B cells and determining immune responses in a hybridoma line specific for the peptide. To test for splicing, an additional four amino acids could be added between the amino acids of the cathepsin D digestion site. This synthetic peptide could then be added to the B cell culture medium, washed well before addition to the hybridomas, and the hybridoma culture media checked for IL-2 responses compared with background, before testing the APCs used for peptide expressed on their surfaces by HPLC and MS.

The work in Chapter 6, as stated above, does not support the circulation of T cells specific for the Goodpasture antigen in normal circulating blood of the mouse. This could be due to lack of sensitivity of the assay, such that only a very few cells would be able to recognise the Goodpasture antigen and that within the experiments described, they were not in contact with the relevant stimulatory peptide. This suggests that additional work could be done to characterise immune responses from $\alpha 3(\text{IV})\text{NC1}$ wild-type mice. However, there is a fundamental difference between the experiments described in Chapter 6 and the data published by Zou *et al.* (3). The cells isolated from healthy volunteers were unprimed, whereas the wild-type mice used in the experiments in Chapter 6 were immunised prior to extraction of the lymph nodes. It would be interesting to see whether the mice had circulating T cells to the Goodpasture antigen without prior immunisation as it is possible that

peripheral tolerance mechanisms were enhanced following immunisation with the Goodpasture antigen in complete Freund's adjuvant. This hypothesis suggests that activated Goodpasture-specific T cells could be subjected to overwhelming regulatory mechanisms resulting in deletion of the T cells and preventing an aberrant immune response. It could also partially explain the lack of immune response to peptides in primed $\alpha 3(\text{IV})\text{NC1}$ WT mice. Certainly, it has been shown that B cell anergy caused by peripheral tolerance may be reversed with appropriate T cell help, causing autoimmunity in otherwise non-autoimmune mice (283), and that multiple species express autoreactive antibodies while in a normal, healthy state (284). The converse may also be true (as hypothesised here) but would require further study. In particular, peripheral tolerance mechanisms controlling T cell activation and the conditions required to delete the resultant clonal expansion of autoreactive T cells, should be investigated.

It would also be interesting to ascertain whether tolerance could be broken in these animals or conversely, if tolerance could be induced in the animals that do not express endogenous $\alpha 3$, inducing tolerance to a foreign antigen. Work by Reynolds *et al.* suggested that tolerance in rats could be induced by intranasal administration of recombinant Goodpasture antigen, resulting in failure to develop autoimmune glomerulonephritis (281). It would be interesting to determine whether the same is true in the transgenic mice used throughout this project. This has implications for treatment of Alport's disease. As described in Chapter 3, Alport's disease occurs when (usually) the $\alpha 5(\text{IV})$ chain is not expressed, resulting in renal failure and the need for transplant. These patients often develop Goodpasture-like glomerulonephritis by reacting to the $\alpha 5(\text{IV})$ within the donor kidney as foreign

(209). If tolerance could be induced in humans, it could prevent allograft rejection in these patients.

This work is different from much of the current studies investigating the role of central tolerance in determining susceptibility to autoimmunity. Much of the published data involves introduction of a foreign antigen into a species in order to determine mechanisms involved in propagation of tolerance – Cibotti's work using mice that express HEL for example (120, 153, 157, 161, 285). In this work, the converse is true. My project aimed to determine differences in immune responses to proteins to which central tolerance is never induced. This approach has also been used to examine some aspects of multiple sclerosis, with the development of myelin-basic protein deficient "shiverer" mice. The genetic defect is an autosomal recessive mutation and symptoms in the form of tremors and convulsions are seen from approximately two weeks of age. The mutation causes severe myelin deficiency in the central nervous system, due to defective myelin formation (286). These mice have been extensively used to examine cell engraftment, migration and myelination with a long term aim of curing or ameliorating symptoms of MS. Recent work has involved prenatal treatment of shiverer mice with stem cells, generating chimeric mice with strong MBP expression throughout the brain and significantly reduced behavioural difficulties (287).

Research more pertinent to my work has been published less recently. Yoshizawa *et al.* showed that BALB/c mice (shiverer mice are on a BALB/c background), after immunisation with MBP do not develop EAE. Experiments using shiverer mice found that there are specific epitopes on the MBP that induce T cell proliferation (residues 59-76 and 89-101) that these T cells are also able to induce

EAE upon injection into normal BALB/c mice (288). It has also been shown that endogenous MBP induced profound inactivation of high avidity clones specific for the immunodominant determinant making that determinant appear cryptic. Immunisation of mice expressing MBP with MBP peptide 79-87 found that only low avidity T cell clones were generated and that these clones would not respond to the whole protein, only to the peptide in recall response assays (289). My data studying immune responses to the Goodpasture antigen corroborates these data as the $\alpha 3$ WT mice did not have an immune response to the antigen, while the $\alpha 3$ KO mice did.

It is difficult to study the effects of tolerance in humans, but one example in which it can be done is with the red blood cell antigen Rhesus D (RhD). Approximately 15% of the Caucasian population lack the RhD protein on a structural lipoprotein within the erythrocyte membrane (290). This provides a rare opportunity to examine the response to RhD both in the presence and absence of tolerance in humans. The most common situation involving this antigen is when a RhD-negative pregnant female carries an RhD-positive foetus causing erythroblastosis fetalis in which antibodies generated in the mother attack the unborn foetus (290). Recent work has examined the T cell recall responses in humans (either following deliberate RhD-immunisation or RhD incompatible pregnancy) using overlapping 15mer peptides in much the same way as the recall responses to $\alpha 3$ (IV)NC1 were determined within Chapters 6 and 7. This work determined that there are 4 peptide sequences in particular that elicit a proliferative T cell response in more than 50% of the humans tested – amino acids 52-66, 97-111, 117-131 and 177-191 (291). It has also been shown that of these peptides the amino acid sequence 52-66 induces IL-10 production and therefore induces peripheral tolerance by suppression of effector T

cell responses (292). Subsequent work has shown that tolerance can be induced to the RhD protein by immunising HLA transgenic mice with intranasal administration of these immunodominant peptides (293). This induction of tolerance following intranasal immunization of antigen has also been successfully demonstrated in WKY rats and the Goodpasture antigen (281), but it remains to be seen how such knowledge can be utilized for the induction of tolerance to the Goodpasture antigen in humans.

To summarise, this project studied immune responses to the Goodpasture antigen. The data found was surprising. It was shown that $\alpha 3$ KO mice generate immune responses to a similar sequence as human Goodpasture patients (these patients express endogenous $\alpha 3$ (IV)), a sequence that should be destroyed by cathepsin D processing mechanisms. These responses were not seen in mice that express endogenous $\alpha 3$ (IV)NC1, suggesting robust tolerance despite sequence differences (mice were immunised with human $\alpha 3$ (IV)NC1). Additional questions now arise: are MHC class II processing pathways different in mouse and man, and do the fine specificities of processing enzymes differ? What role does peripheral tolerance play in regulating autoreactive T cells and can it be upregulated in response to a CD4 T cell autoimmune reaction? Can tolerance be induced to specific antigens in order to prevent or treat autoimmunity?

The work described here provides a solid foundation for further work to be undertaken to explore mechanisms preventing and causing Goodpasture's disease, and these mechanisms may be applicable to other autoimmune diseases. In particular, mechanisms controlling tolerance could be further investigated, since the

transgenic mice developed are equipped to compare responses in the presence and absence of central tolerance.

Appendix I - α 3(IV)NC1 overlapping peptide sequences
and peptide sets

Peptide name	Amino acid start position	Amino acid finish position	Sequence (FASTA format)
α 3(IV)NC1 -9-5	-9	5	GLKGKRGDSGSPATW
α 3(IV)NC1 -4-10	-4	10	RGDSGSPATWTTRGF
α 3(IV)NC1 1-15	1	15	SPATWTTRGFVFTRH
α 3(IV)NC1 6-20	6	20	TTRGFVFTRHSQTTA
α 3(IV)NC1 11-25	11	25	VFTRHSQTTAIPSCP
α 3(IV)NC1 16-30	16	30	SQTTAIPSCPEGTVP
α 3(IV)NC1 21-35	21	35	IPSCPEGTVPLYSGF
α 3(IV)NC1 26-40	26	40	EGTVPLYSGFSFLFV
α 3(IV)NC1 31-45	31	45	LYSGFSFLFVQGNQR
α 3(IV)NC1 36-50	36	50	SFLFVQGNQRAHGQD
α 3(IV)NC1 41-55	41	55	QGNQRAHGQDLGTLG
α 3(IV)NC1 46-60	46	60	AHGQDLGTLGSCLQR
α 3(IV)NC1 51-65	51	65	LGTLGSCCLRFTTMP
α 3(IV)NC1 56-70	56	70	SCLRFTTMPFLFCN
α 3(IV)NC1 61-75	61	75	FTTMPFLFCNVNDVC
α 3(IV)NC1 66-80	66	80	FLFCNVNDVCNFAFR
α 3(IV)NC1 71-85	71	85	VNDVCNFAFRNDYSY
α 3(IV)NC1 76-90	76	90	NFAFRNDYSYWLSTP

Peptide name	Amino acid start position	Amino acid finish position	Sequence (FASTA format)
α 3(IV)NC1 81-95	81	95	NDYSYWLSTPALMPM
α 3(IV)NC1 86-100	86	100	WLSTPALMPMNMAPI
α 3(IV)NC1 91-105	91	105	ALMPMNMAPITGRAL
α 3(IV)NC1 96-110	96	110	NMAPITGRALEPYIS
α 3(IV)NC1 101-115	101	115	TGRALEPYISRCTVC
α 3(IV)NC1 106-120	106	120	EPYISRCTVCEGPAI
α 3(IV)NC1 111-125	111	125	RCTVCEGPAIAIAVH
α 3(IV)NC1 116-130	116	130	EGPAIAIAVHSQTTD
α 3(IV)NC1 121-135	121	135	AIAVHSQTTDIPPCP
α 3(IV)NC1 126-140	126	140	SQTTDIPPCPHGWIS
α 3(IV)NC1 131-145	131	145	IPPCPHGWISLWKGf
α 3(IV)NC1 136-150	136	150	HGWISLWKGFSFIMF
α 3(IV)NC1 141-155	141	155	LWKGFSFIMFTSAGS
α 3(IV)NC1 146-160	146	160	SFIMFTSAGSEGTGQ
α 3(IV)NC1 151-165	151	165	TSAGSEGTGQALASP
α 3(IV)NC1 156-170	156	170	EGTGQALASPGSCLE
α 3(IV)NC1 161-175	161	175	ALASPGSCLEEFRAS
α 3(IV)NC1 166-180	166	180	GSCLEEFRASPFLEC
α 3(IV)NC1 171-185	171	185	EFRASPFLECHGRGT
α 3(IV)NC1 176-190	176	190	PFLECHGRGTCNYYS
α 3(IV)NC1 181-195	181	195	HGRGTCNYYSNSYSF

Peptide name	Amino acid start position	Amino acid finish position	Sequence (FASTA format)
$\alpha 3(\text{IV})\text{NC1 } 186\text{-}200$	186	200	CNYYSNSYSFWLASL
$\alpha 3(\text{IV})\text{NC1 } 191\text{-}205$	191	205	NSYSFWLASLNPERM
$\alpha 3(\text{IV})\text{NC1 } 196\text{-}210$	196	210	WLASLNPERMFRKPI
$\alpha 3(\text{IV})\text{NC1 } 201\text{-}215$	201	215	NPERMFRKPISTVK
$\alpha 3(\text{IV})\text{NC1 } 206\text{-}220$	206	220	FRKPISTVKAGELE
$\alpha 3(\text{IV})\text{NC1 } 211\text{-}225$	211	225	PSTVKAGELEKIISR
$\alpha 3(\text{IV})\text{NC1 } 216\text{-}230$	216	230	AGELEKIISRCQVCM
$\alpha 3(\text{IV})\text{NC1 } 221\text{-}235$	221	235	KIISRCQVCMKKRH
AS345	65	82	SPFLFCNVNDVCNFA SRND

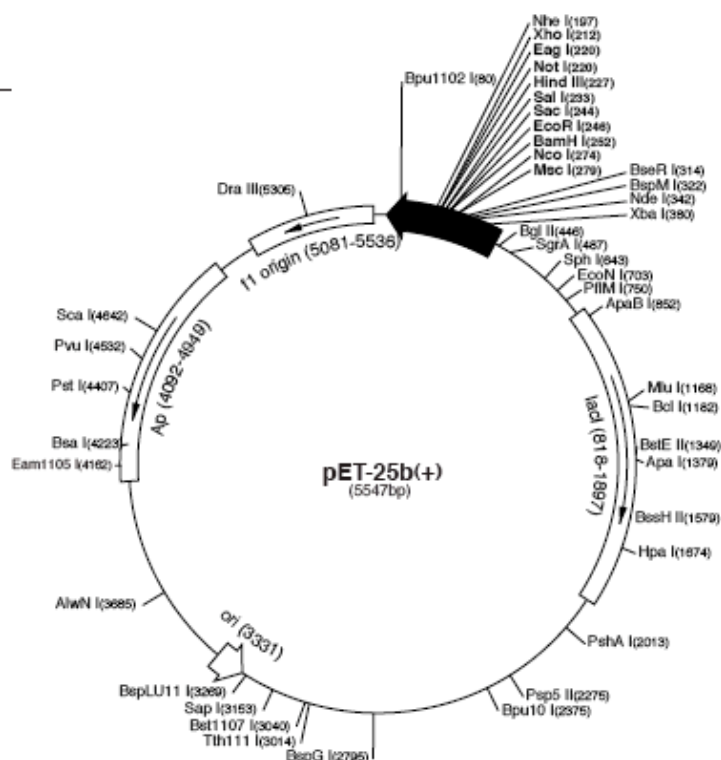
Name of Set	Start peptide	Last peptide	No. peptides
s1	α 3(IV)NC1 -9-5	α 3(IV)NC1 11-25	5
s2	α 3(IV)NC1 6-20	α 3(IV)NC1 26-40	5
s3	α 3(IV)NC1 21-35	α 3(IV)NC1 41-55	5
s4	α 3(IV)NC1 36-50	α 3(IV)NC1 56-70	5
s5	α 3(IV)NC1 51-65	α 3(IV)NC1 71-85	5
s6	α 3(IV)NC1 66-80	α 3(IV)NC1 86-100	5
s7	α 3(IV)NC1 81-95	α 3(IV)NC1 101-115	5
s8	α 3(IV)NC1 96-110	α 3(IV)NC1 116-130	5
s9	α 3(IV)NC1 111-125	α 3(IV)NC1 131-145	5
s10	α 3(IV)NC1 126-140	α 3(IV)NC1 146-160	5
s11	α 3(IV)NC1 141-155	α 3(IV)NC1 161-175	5
s12	α 3(IV)NC1 156-170	α 3(IV)NC1 176-190	5
s13	α 3(IV)NC1 171-185	α 3(IV)NC1 191-205	5
s14	α 3(IV)NC1 186-200	α 3(IV)NC1 206-220	5
s15	α 3(IV)NC1 201-215	α 3(IV)NC1 221-235	5

Appendix II – Restriction Maps of plasmid vectors

pET25b, bacterial expression vector

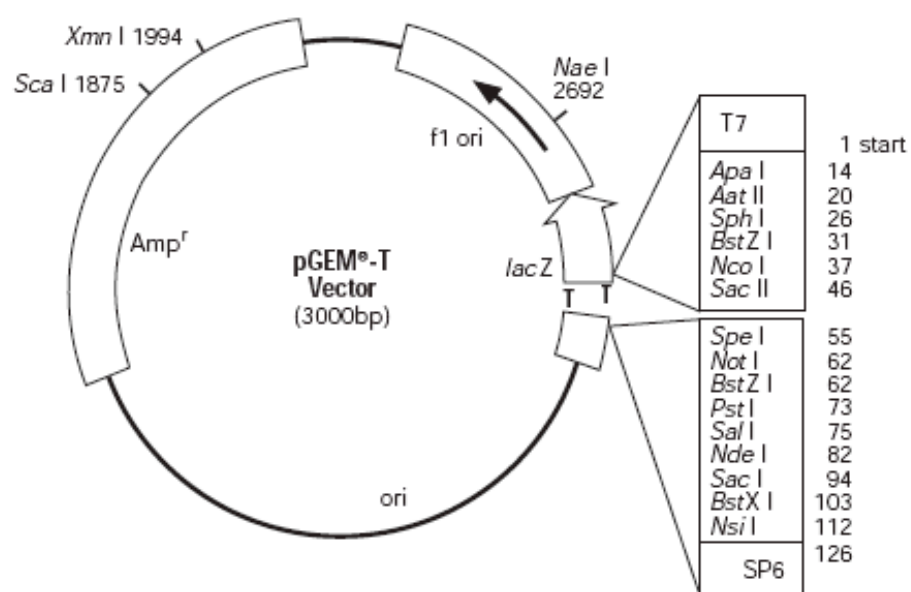
pET-25b(+) sequence landmarks

T7 promoter	415-431
T7 transcription start	414
<i>pelB</i> coding sequence	278-343
Multiple cloning sites (<i>Nco</i> I - <i>Xba</i> I)	212-279
HSV-Tag coding sequence	164-199
His-Tag coding sequence	140-157
T7 terminator	26-72
<i>lacI</i> coding sequence	818-1897
pBR322 origin	3331
<i>bla</i> coding sequence	4092-4949
<i>ori</i> origin	5081-5536

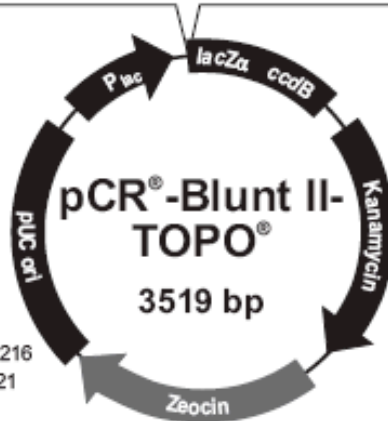
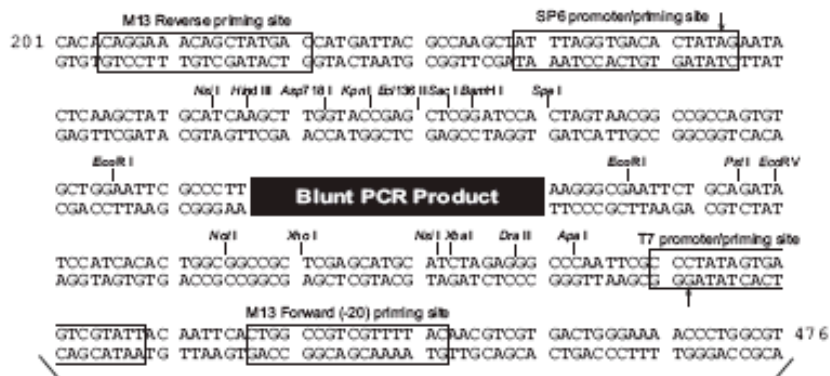


pGEM-T, bacterial cloning vector

B. pGEM®-T Vector Map and Sequence Reference Points



TOPO blunt cloning vector, pCR-BLUNT II-TOPO



Comments for pCR®-Blunt II-TOPO®
3519 nucleotides

lac promoter/operator region: bases 95-216

M13 Reverse priming site: bases 205-221

LacZ-alpha ORF: bases 217-576

SP6 promoter priming site: bases 239-256

Multiple Cloning Site: bases 269-399

TOPO®-Cloning site: bases 336-337

T7 promoter priming site: bases 406-425

M13 (-20) Forward priming site: bases 433-448

Fusion joint: bases 577-585

ccdB lethal gene ORF: bases 586-888

kan gene: bases 1099-2031

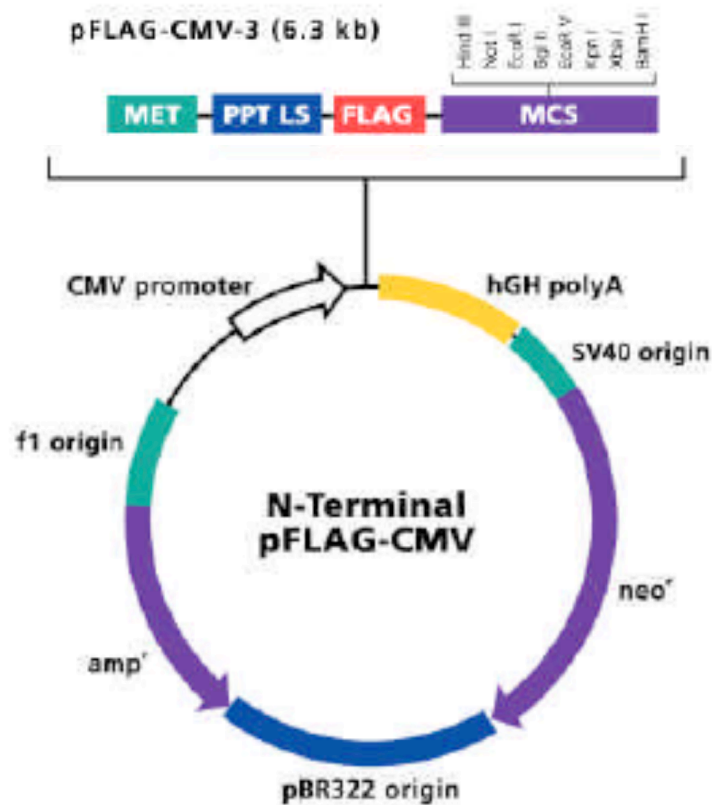
kan promoter: bases 1099-1236

Kanamycin resistance gene ORF: bases 1237-2031

Zeocin resistance ORF: bases 2238-2612

pUC origin: bases 2724-3397

*pFLAG-CMV-3 Vector for mammalian cell transfection and secretion
of protein*



Multiple Cloning Site

(pFLAG-CMV-3* and pFLAG-CMV-4)

FLAG Peptide Sequence														
Met*	Asp	Tyr	Lys	Asp	Asp	Asp	Asp	Lys	NotI			EcoRI		
ATG	GAC	TAC	AAA	GAC	GAT	GAC	GAC	AAG	CTT	GCG	GCC	GCG	AAT	TCA
TAC	CTG	ATG	TTT	CTG	CTA	CTG	CTG	UTC	GAA	CGC	CGG	CGC	TTA	AGT
									HindII					
BglII				EcoRV			KpnI		XbaI			BamHI		
TCG	ATA	GAT	CTG	ATA	TCG	GTA	CCA	GTC	GAC	TCI	AGA	GGA	TCC	CGG
AGC	TAT	CTA	GAC	TAT	AGC	CAT	GCT	CAG	CTG	AGA	TCT	CTT	AGG	CCC

*For pFLAG-CMV-3, the Met-preprotrypsin leader sequence (PPTLS) precedes the FLAG coding sequence.

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